TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

MARIANNA GRANATIER

CULTIVATION OF *SCENEDESMUS ACUMINATUS* IN OPEN PONDS AND SIMULTANEOUS NUTRIENT REMOVAL FROM SOURCE SEPARATED URINE

Master of Science Thesis

Examiners: Professor Jukka Rintala, Assistant professor Marika Kokko and Doctor Pritha Chatterjee
Examiners and topic approved on 27th of September 2017

ABSTRACT

MARIANNA GRANATIER: Cultivation of *Scenedesmus acuminatus* in open ponds and simultaneous nutrient removal from source separated urine
Tampere University of Technology
Master of Science Thesis, 59 pages
December 2017
Master's Degree Programme in Bioengineering
Major: Bioengineering
Examiner: Professor Jukka Rintala, Assistant professor Marika Kokko, Doctor Pritha Chatterjee

Keywords: source separated urine, microalgae, pilot raceway pond

Increasing human population calls for food security and providing enough food is coupled with frequent use of chemical fertilizers in agriculture. Phosphorus and nitrogen are one of the most essential nutrients for living organisms and the main components of chemical fertilizers. Currently, the only way to obtain phosphorus, is mining it from finite phosphate rock. However, majority of the phosphorus is after one-time use of fertilizer irreversibly wasted and drained into the natural water bodies causing uncontrollable microalgal bloom.

In detail, urine is a source that concentrates high amount of phosphorus and nitrogen. Using source separated urine as a natural fertilizer for microalgal cultivation represents attractive and promising method to recover phosphorus and nitrogen. This study is the first pilot study reported in the literature that tests source separated urine as a growth medium for microalgal cultivation in open ponds with potential to recover the nutrients. *Scenedesmus acuminatus* was cultivated for 94 days in two different set ups: a) batch set up with 400 l working volume and 20x diluted urine; and b) semi-continuous set up with 2000 l working volume and 20x or 15x diluted urine. Results showed that *S. acuminatus* can achieve high yields when cultivated in 20x (2.3 g of VSS/ l of pond volume). Moreover, results showed that semi-continuous cultivation of microalgae grown on 15x and 20x diluted urine is sustainable and feasible (maximum microalgal yield was 0.45 g of VSS/ l of pond volume). The analysis of nutrient concentration showed that phosphorus and nitrogen were removed from 20x and 15x diluted urine, but further measurements are needed to exactly determine how much of phosphorus and nitrogen were recovered by *S. acuminatus*.

PREFACE

At the end of my Mater´s studies, writing the last missing piece in this thesis, I would like to express one endless thank you to everybody who helped me during this journey.

In particular, I thank to professor Jukka Rintala and Marika Kokko for their guidance and for the opportunity to be a part of this amazing project and team. I thank to Pritha Chatterjee and Praveen Ramasamy for their patience, answers to my questions and for all that knowledge they gave me. I would like to thank to Réka- Hajdu Rahkama for her valuable everyday company and so many happy moments. Last but not least, I thank to Alireza Changizi who stand beside me in each moment of this long process.

Tampere, 22.11.2017

Marianna Granatier

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

<>	more than, less than
%	percent
~	approximately
°C	grade Centigrade
µm	micrometer
µM	micromolar
15x	15 times
20x	20 times
ATP	adenosine-triphosphate
Ca^{2+}	calcium cation
CO_2	carbon dioxide
COD	chemical oxygen demand
DO	dissolved oxygen
DWW	domestic wastewater
g	gram
HU	human urine
K	potassium
K^+	potassium cation
kg	kilogram
L / l	liter
M	meter
mg/l	milligram per litre
Mg^{2+}	magnesium cation
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MT	mega tons
N	nitrogen
Na^+	sodium cation
NH_3	ammonia
NH_4^+	ammonium
NH_4-N	ammonium nitrogen
nm	nanometer
NO_2^-	nitrite
NO_3^-	nitrate
N_{tot}	total nitrogen
OD	optical density
P	phosphorus
PBR	photobioreactor
P_{cc}	phosphorus critical concentration
P_{extra}	extracellular phosphorus concentration
P_{intra}	intracellular phosphorus concentration
PO_4^-	phosphate
P_{tot}	total phosphorus
rpm	rotation per minute
RwP	raceway pond
SCOD	soluble chemical oxygen demand
SSU	source separated urine
SU	synthetic urine
T	temperature
TSS	total suspended solids
UDT	urine diversion toilet
VSS	volatile suspended solids

1. INTRODUCTION

One of the most significant global concerns is the growing population and food safety that should be provided. Nowadays, the only way how to provide enough feed for humans is the use of fertilizers in agriculture. The main component of chemical fertilizers and an essential element for all organisms is phosphorus. Mining of the finite phosphate rock is currently only way how to obtain phosphorus in large amounts. Moreover, the scarcity of the phosphate rock is more and more real (Melia et al., 2017). Nitrogen is along with phosphorus another essential element for life, and it is used in the chemical fertilizers as well. Production of nitrogen fertilizers is based on nitrogen fixation from the atmosphere. Therefore, there is no threat of nitrogen depletion, but its fixation is costly, and it increases greenhouse gas emissions due to the use of fossil fuels (Ledezma et al., 2015; Wang et al., 2017).

Another problem arising from the applied fertilizers is their one-time use and their abundant release into the environment causing pollution of natural water bodies (Ledezma et al., 2015; Roy, 2017). Majority of these key nutrients end as a waste. In particular, they are excreted from the human body in the form of urine and feces. Urine contributes only 1% of the domestic wastewater (DWW), but its rich composition in phosphorus (50% from the DWW) and nitrogen (80% from the DWW) makes it the best candidate for nutrient recovery (Chang et al., 2013).

At the moment, only a few methods can be applied to nutrient recovery from the urine. One of them is struvite precipitation when by adding magnesium, phosphorus and ammonia start to precipitate and consequently they can be removed from the urine. Another method focusing on phosphorus removal is the calcium phosphate precipitation. Nitrogen is removed by ammonia stripping or ion exchange. Despite the applicability of the mentioned methods, they still bring challenges, like precipitation in the pipelines or low affinity for the exchanger, that make the whole process of recovery costly (Cieślik and Konieczka, 2017; Carey et al., 2016; Wang et al., 2017).

Using urine directly as a fertilizer is known for a very long time (Roy, 2017). However, more and more studies are discussing using urine as a feed for microalgae. Cultivation of microalgae in urine brings advantages like a free source of water, phosphorus and nitrogen for microalgae, a decreased water pollution and greenhouse gas emission (Patel et al., 2017). They can be cultivated in the open ponds or closed photobioreactors, and they do not require arable land (Cuellar-Bermudez et al., 2017). The composition of microalgae is full of proteins, carbohydrates, lipids as well as fatty acids or pigments and currently

they are widely used as human health supplements or animal feed (e.g., *Chlorella* sp. or *Spirulina* sp.) (Spolaore et al., 2006).

The primary goal of this work was to use the source separated urine as a feed for fresh-water microalgae *Scenedesmus acuminatus* with the purpose of nitrogen and phosphorus recovery. The uniqueness of this project consists in the testing of microalgal cultivation in open ponds in a greenhouse in a northern latitude.

2. NUTRIENT RECOVERY

In today's world, with increasing population, the demand for food production is increasing exponentially. To satisfy basic human needs and live in a safe environment, it is necessary to use the natural resources sustainably and at the same time recycle the created waste (Carey et al., 2016). In particular, food security needs a continuous supply of agricultural fertilizers. It is estimated that the agricultural market comprises annual production of 176 MT of fertilizer costing more than $130 billion and it will keep growing in the future (Jönsson et al., 2013). The most significant concern is the one-time use of fertilizers and their consequent discharge into the environment. Their production is strongly dependent on the limited mineral reserves and non-renewable energy sources. Fertilizers are characterized by high content of macronutrients essential for plant growth: nitrogen (N), phosphorus (P) and potassium (K) (Ledezma et al., 2015).

2.1 Phosphorus

One of the most essential nutrients for humans, animals, and plants is phosphorus (P). It is crucial for cell development and energy storage in a living organism. It can be found as inorganic phosphate ion (PO_4^-) in the soil, water, and sediments. Figure 1 represents phosphorus cycle in nature. Firstly, rain and harsh weather cause the release of inorganic P from the rocks and its distribution in soil and water. Then plants, which can be eaten by animals, can uptake P from the soil. When animals and plants die, they decay, and the organic P is returned to the soil. Sometimes, a bacterial activity can decompose organic P into inorganic form. At the same time, P can be flushed from the soil to the water deposits (Childers et al., 2011).

In addition, human activity significantly affects the natural cycle of phosphorus (Figure 1). To meet the ever-increasing demand for food, plants and crops are harvested giving no space to the natural return of the P into the soil. Therefore, farmers need to replenish P by using fertilizers. As a consequence, phosphorus is being widely used in agriculture as a component of fertilizers (Childers et al., 2011). The only way to obtain phosphorus in large scale for fertilizer production is to extract it from phosphate rocks. Statistics show that only 20% of mined P is used efficiently. Rest of the 80% is wasted (Solovchenko et al., 2016). That is the point where the concerns arise. Firstly, the phosphate source is not distributed evenly around the world. The most prominent leaders in phosphate rock mining are United States, China, Morocco, Jordan, South Africa and Algeria. Secondly, P is a non-renewable source, and it is estimated that majority of the phosphate rock will be exploited during this century. Lastly, a great amount of the P is dispersed and lost in water systems and landfills without any further recovery (Solovchenko et al., 2016).

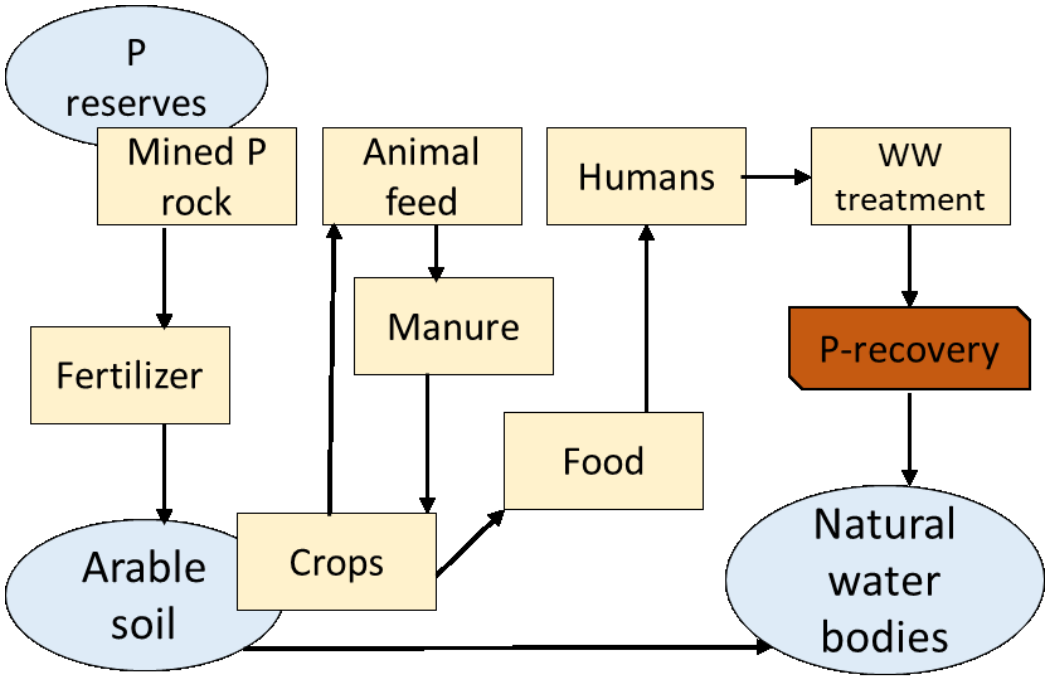

Figure 1. Major global phosphorus flow: from mining to discharge into natural water sources (Modified from (Melia et al., 2017)).

2.2 Nitrogen

Along with P, another essential nutrient is nitrogen (N). It is a colorless and odorless gaseous element forming 78% of the Earth´s atmosphere. N is necessary for amino acids and DNA formation in each living cell. It has an important role in chlorophyll synthesis in the photosynthetic organisms. The nitrogen cycle of nature is, therefore, encompassed between atmosphere, land and the living organisms. The first step is the fixation of atmospheric nitrogen (N_2) by soil bacteria (in the form of ammonium ion NH_4^+), light (ammonia NH_3 or nitrate NO_3^-) or human activity (NH_3). Fixed N_2 is taken by plants, plants are eaten by animals, and when animals die or excrete, the nitrogen enters the soil in organic form. Decomposing bacteria in the soil convert organic nitrogen into NH_3, which is further processed through nitrification process. As a result, nitrite (NO_2^-) and nitrate (NO_3^-) are formed and again taken up by plants. The cycle is completed when denitrification of NO_3^- occurs, and the gaseous N_2 is released by denitrifying bacteria into the atmosphere (Canfield et al., 2010).

From the nitrogen cycle, it can be seen that N is essential for growth of crops. Hence, it is widely used for the production of fertilizers (Carey et al., 2016). For large scale production of fertilizers, nitrogen needs to be fixed from the atmosphere by the Haber-Bosch process. The synthesis of ammonia is based on the reaction of hydrogen and nitrogen under high pressure, moderate temperature and catalyst activity (Milton et al., 2017). Nitrogen is abundant on the earth, and there is no scarcity of the nitrogen resource. Nevertheless, it is important to think about the recovery mainly because of the environmental

benefits (decreasing water deterioration and greenhouse gas emission coupled with the burning of fossil fuels) and economic issues like overall decrease of energy consumption (Maurer et al., 2017).

2.3 Effects of phosphorus and nitrogen in wastewater streams and their recovery

As mentioned earlier, human activity significantly influences the natural cycle of P and N. With increasing population and simultaneous increasing demand for food, P and N are irreversibly getting lost in domestic waste (Wang et al., 2013). In particular, domestic wastewater (DWW) contains a big portion of P and N. DWW combines water that comes from homes, commercial institutions and industrial facilities. DWW is generated by bathing, washing and toilet flushing (Rawat et al., 2011). Urine is the most significant fraction of DWW containing P and N. Detailed statistics show that one person can produce around 1.5 L of urine/ day what counts for the production of 2 - 4 kg of N/person per year and 0.2 - 0.37 kg of P/ person per year only in the urine (Kvarnström et al., 2006). Even though urine contributes only 1% of DWW volume, it carries the biggest load of the nutrients from DWW: 80% of nitrogen, 50% of phosphorus and 90% of potassium. Moreover, human urine contains trace elements (e.g., Zn, Cu, Fe) and it is usually free from heavy metals, hazardous compounds, and pathogens (Chang et al.,2013). On the other hand, if the urine is further used, it is important to consider that urine is the primary medium of micropollutants (pharmaceutical and hormone) excretion (Maurer et al., 2006). Micropollutants can have adverse effects on aquatic organisms and human health. They accumulate inside the body and they can potentially act like endocrine system disruptors, or they can possibly develop antibiotic resistance (Yang et al., 2017; Li et al., 2015). However, the scientific literature is still lacking statistically significant evaluation of the effects of micropollutants present in urine on living organism (Maurer et al., 2006).

Proper treatment of DWW and thus urine, is essential not only for saving the water resources but also for nutrient recovery and consequent nutrient´s source preservation. Unfortunately, releasing domestic wastewater with urine directly into the natural water bodies is still common phenomenon all around the world. As a result, the fresh waters are rich in nutrients (P, N, K) what causes eutrophication. In other words, increased availability of nutrients promotes excessive growth of water plants and algae. Dense algal vegetation limits the penetration of the light, depletes dissolved carbon, dramatically increases pH and can release toxins. Consequently, all these changes lead to the extinction of the animals and vegetation and can negatively affect humans (Chislock et al., 2013). Moreover, it reduces the amount of water directly available for human use (only 0.75% of total water on the Earth is available for human consumption) (Cuellar-Bermudez et al., 2017).

Treating the urine separately from the wastewater could represent a promising solution for nutrient recovery. However, it comprises several challenges that begin in the households because the technology for urine treatment available for everyday users is still expensive and inefficient (Maurer et al., 2006). Nevertheless, there are already some industrially applied methods for P and N recovery, for example, precipitation method for P in Japanese factories. Recovery methods are pointed for digester supernatant treatment, but they could be used for urine treatment as well (Cieślik and Konieczka, 2017; Maurer et al., 2006).

2.3.1 Phosphorus recovery from urine

Precipitation is the most commonly used technique for phosphorus recovery. One of the preferred precipitated minerals is magnesium ammonium phosphate ($MgNH_4PO_4.6H_2O$), shortly called struvite precipitation. The purpose of this technique is to remove ammonia and phosphorus in solid form from the wastewater. Removing two main nutrients from the wastewater at the same time is a big advantage, and the obtained products could be further used as a fertilizer with a slow release of nutrients (Maurer et al., 2006). The significant advantage of precipitation is that the precipitated crystals contain a minimal amount of impurities. Additionally, the solubility of the struvite is very low and therefore the potential pollution of the environment, when excess struvite is used, is low as well. On the other hand, the drawback is the addition of the chemicals ($MgCl_2$ or MgO) for reaction initiation and uncontrolled precipitation in the pipelines of the reactor. These drawbacks make the whole process of recovery costly, and the price for the product becomes three times higher than for the traditional fertilizer (Cieślik and Konieczka, 2017).

Precipitation of calcium phosphates (Ca-P) has more potential for commercial use than struvite. The reason is the broader applicability of Ca-P in different industrial branches in comparison to the struvite. Namely, hydroxyapatite strongly mimics the composition of natural phosphate rock. Thus, it could be used as a secondary source of P. However, the precipitation of hydroxyapatite comprises the same challenges as struvite precipitation (Melia et al., 2017).

2.3.2 Nitrogen recovery from urine

Several options for N recovery from urine have been suggested like ammonia (NH_3) stripping and distillation, ion exchange or microbial electrochemical technologies (Maurer et al., 2006).

The most common technology with high recovery efficiency (~ 98%) is NH_3 stripping and distillation, especially when the NH_3 concentration in the urine is very high (NH_4-N > 2000 mg/l). Ammonia stripping requires pH (> 9.5) and temperature (> 80 °C) adjust-

ment before the recovery. A detailed process of ammonia stripping consists of alkali addition when the ammonium ion (NH_4^+) is transformed to NH_3 which volatilizes. Consequently, volatile NH_3 can be stripped from the urine into the air, which is then passed through an acidic solution. As the last step before final fertilizer production, NH_3 is absorbed and recovered through condensation, absorption or oxidation. NH_3 stripping, due to its cost-effectiveness and ease of control, is used for treating not only urine but also wastewater as such (Carey et al., 2016; Zhu et al., 2017).

Ion exchange is based on the principle of adsorption. NH_4^+ is adsorbed by cation exchanger which is made from the natural zeolites or resins. Zeolites have a high affinity for NH_4^+, but low capacity for NH_4^+ concentration and their recovery is energetically inefficient. On the contrary, resins have a high capacity but low affinity for NH_4^+ what causes that other cations (e.g., Ca^{2+} or Mg^{2+}) are preferred to be bonded on the resin instead of NH_4^+ (Z. Wang et al., 2017). Some researchers have shown the possibility of the combination of ion exchange with struvite precipitation (P-recovery) (Maurer et al., 2006). The potential of using ion exchange for NH_4^+ recovery from urine relies on the fact that urea present in urine is spontaneously hydrolyzed to NH_4^+. Despite that, ion exchange for nutrient recovery from urine is not widely studied yet (Tarpeh et al., 2017). The literature reported the study for urine treatment with the concentration of approximately 5000 mg of N/l of urine using clinoptilolite (natural zeolite) and the removal efficiency of ammonium reached 84 % (Baykal et al., 2009).

A novel method of microbial fuel cells (MFC) and microbial electrolysis cells (MEC) describes NH_4^+ recovery from wastewaters rich in ammonia. NH_4^+ ions migrate through the cation-exchange membrane, after which they can be recovered. The advantage of MFC compared to the previous methods is that it requires less energy input, but its application is still only on the laboratory scale due to several challenges. The main two challenges are low ionic conductivity and low buffering capacity of real urine (Ledezma et al., 2015).

3. ENHANCED NUTRIENT RECOVERY FROM SOURCE SEPARATED URINE

3.1.1 Source separation of urine

The urine separation from feces, also called urine diversion, starts exactly at the point of their production. Urine is separated by urine diverting (separating) toilets (UDT) or urinals. In this way, the dilution of urine with any other wastewater stream could be avoided (Kvarnström et al., 2006).

UDT is specially designed toilet (Figure 2) that has the bowl separated into two sections: one for urine and the other for feces collection. Urine and feces may be flushed with water (urine diversion flush toilets) or may not be (dry toilets). In both cases, urine and feces are collected in different storage tanks, but despite that, there could be still space for cross-contamination. New designs of UDT involve pedestal and squatter toilets suitable for water and tissue paper personal cleansing (Kvarnström et al., 2006; Simha and Ganesapillai, 2017). UDT could be built in the rural and urban areas no matter what is the population density, in the regions with insufficient wastewater management but also in the areas of well-developed water supply and pipeline. The primary purpose of UDT is to provide proper sanitation and get quickly available fertilizer (Kvarnström et al., 2006).

UDT is still not a well-known term in the society, but some parts of the world such as El Salvador, Dongsheng and Nanning Guangxi in China, Nacka in Sweden, Sneek in Netherland or Eschborn in Germany took action and implemented the use of UDT in everyday life (Kvarnström et al., 2006; Tuantet et al., 2014). Using UDT represents a revolutionary approach to urine treatment and nutrient recovery. Building UDT could be more economical than expanding or renewing already existing treatment plants. Nonetheless, some challenges make the large-scale implementation of UDT in the world more difficult, no matter if it is a newly built area or area with already existing infrastructure. The major challenge is the education and awareness of the population ranging from the standard UDT user, stakeholders providing UDT, infrastructure and service providers to politicians. UDT require more caring and different sanitation approach than regular toilets. Moreover, UDT is linked to the manipulation of the urine and feces, and this is something that many people do not want to deal with. The UDT includes the knowledge about safe ways of urine (and feces) recycling. The urban planning should be taken into the account since it is preferable that the urine is not transported far away, but instead, it is used in the location nearby the urine generation area. All the challenges mentioned above call for institutional and political support, relevant policies and legislation (Kvarnström et al., 2006).

Figure 2. Urine diversion flush toilet for urine and feces separation (Adapted from (ABC Science, 2012)).

3.1.2 Ways of treating the source-separated urine

It is recommendable to treat the source separated urine before agricultural use. The reason is that the urine itself is a fast-acting fertilizer and it requires careful handling. Otherwise, the possible adverse urine impacts can increase soil conductivity, pH, and salinity as well as lower crop yield. Untreated urine can spread pathogens and micropollutants into the environment, and it causes odor formation, CO_2 and NH_3 volatilization (Ledezma et al., 2015; Simha and Ganesapillai, 2017). This chapter summarizes some of the typical urine pretreatments. In addition, subchapters 2.3.1 and 2.3.2 describe methods for urine treatment combined with nutrient recovery.

Hygienisation

Urine can contain pathogens mainly if it comes from unhealthy individuals. In addition, fecal contamination can increase the content of microbes in urine. There is no detailed study of exposition routes and effects of these microbes on humans, but despite that, the health risks should be eliminated. Storing the source separated urine (SSU) is the best available method for urine hygienisation at the moment. The storage time depends on the pH, temperature and the scale of the system but overall six months period at the temperature >20°C and elevated pH ~9 should be enough to destroy pathogens (Maurer et al.,

2006; Simha and Ganesapillai, 2017). If the stored urine is intended to be used only for the single household where it was collected, then one-month storage is believed to be enough (Langergraber and Muellegger, 2005). Hydrolysis of urea by bacterial urease elevates pH which is beneficial for pathogen elimination, but on the other hand, it causes the precipitation of phosphorus and volatilization of ammonia which are undesirable effects due to loss of nutrients (Maurer et al., 2006; Simha and Ganesapillai, 2017).

Bacteria, protozoa, and viruses will naturally die over the time. However, bacteria can survive if the living conditions are favorable. For instance, the optimal temperature for most of the microorganisms is around 25 – 30 °C and optimal pH is about 7. Therefore, elevated temperatures (~40- 50°C) and pH (9-12) or addition of ammonia will help to destroy microorganisms completely (Schönning and Senström, 2004).

For large scale hygienisation and storage of urine, usually permanent tanks made from either concrete or plastic are used. For small-scale, small plastic tanks for facilitated transfer are recommended (Kvarnström et al., 2006).

Stabilization

As it was already mentioned, urine can contain microorganisms. Microbial activity is responsible for degradation of organic matter, hydrolyzation of urea with volatilization of NH_3 and salt precipitation resulting in urine degradation. Therefore, the main purpose of urine stabilization is the inhibition of bacterial growth and avoidance of urine deterioration. Acidification and nitrification are possible ways for urine stabilization. Both methods lower the pH of urine. pH of urine can decrease below 4 by acidification, and such a low pH can make pharmaceuticals less reactive in the urine. Nitrification of urine produces either ammonium-nitrate (1:1) or ammonium-nitrite (1:1), but it never converts all ammonia in the urine into nitrite or nitrate (Maurer et al., 2006).

Volume reduction

Volume reduction includes evaporation, freeze-thaw and reverse osmosis. The main benefit of volume reduction is nutrient concentration and easiness of handling (Maurer et al., 2006).

Evaporation of urine is the easiest way to reduce and recover water from urine. Nevertheless, evaporation is coupled with a) ammonia loss that could be solved by the acidification of urine or by working with non-hydrolyzed urine and energy recovery; b) energy demand that could be diminished by energy recovery (Maurer et al., 2006). An evaporation method is not part of the industrial scale yet, but there are laboratories, which are focusing on this topic. For example, Antonini et al. (2012) tested the pilot scale of solar thermal evaporation of human urine (Antonini et al., 2012).

Freezing the urine can concentrate around 80% of nutrients in 25% of the original volume of the urine. This method could be the option for places with cold climate since it will not require extra energy. Similarly, like evaporation, freeze-thaw has only been reported in laboratory scale (Maurer et al., 2006). Ganrot et al. (2007) used frozen urine after thawing along with ion exchange and struvite precipitation to recover N and P. Maximum P recovery that they achieved, was 100 % (in the form of struvite) and maximum recovery of N was 60% (Ganrot et al., 2007).

Reverse osmosis (RO) can recover around 70% of ammonium and 73% of phosphate from the acidified urine. The efficiency of RO depends on the pH because osmosis membrane can retain NH_4^+ better than NH_3. Moreover, the membrane can separate micropollutants from nutrients. The limiting factor in RO is the precipitation of salts on the membrane (Maurer et al., 2006). Scientific literature does not refer to an industrial scale or actual urine treatment, but for example, in the study of Grundestam and Hellström (2007), RO was used for wastewater treatment (Grundestam and Hellström, 2007).

Nutrient recovery from urine by microalgae cultivation

Methods for urine treatment presented in this chapter so far are all physicochemical operations. Nevertheless, a new alternative to combine urine treatment with microalgae shows promising results. Microalgae are an efficient, economical and environmentally acceptable tool for urine treatment (Tuantet et al., 2014; Tuantet et al., 2014). This topic is further discussed in the Chapter 4.

4. MICROALGAE CULTIVATION

The effort of environmental research is to create new ways of nutrient recovery that will be running on renewable energy and will not represent unacceptable health issues. Cultivation of crops in agriculture and using urine as a traditional fertilizer is known for a very long time (Roy, 2017). However, thanks to its composition it could be directly used as a growth medium for microalgae cultivation which could be consequently used as a base for different products (feed, fertilizer, biofuel) (Chang et al., 2013). There are multiple benefits of growing microalgae on urine. Firstly, urine can be a free and continuous source of P, N and water for microalgal growth. Secondly, grown microalgal biomass can have a further use (biodiesel, production of methane and fertilizer) and cultivation of microalgae in urine as such do not need arable land (Cuellar-Bermudez et al., 2017). Thirdly, microalgae produce oxygen (O_2) and can reduce chemical and biochemical oxygen demand of the incoming waste stream. Fourthly, microalgae can reduce greenhouse gas emissions by consuming CO_2. Lastly, they can remove coliform bacteria and heavy metals potentially present in urine (Patel et al., 2017; Trivedi et al., 2015).

Microalgae, also known as microphytes, are unicellular, microscopic algae that can live like single cells or form colonies. Their natural living environment consists mostly of freshwater or marine systems, but some of them can be classified as terrestrial algae. Back to the history, microalgae appeared on the earth billions of years ago, and despite diverse and dramatic environmental changes, they survived until today. There is an estimation that more than 50, 000 algal species exist in the world (Cuellar-Bermudez et al., 2017; Patel et al., 2017). Microalgae are majorly photoautotrophic organisms. They utilize light and carbon dioxide (CO_2) and transform it into the lipids, proteins, and carbohydrates stored in the microalgal biomass. During the photosynthesis, light and CO_2 are absorbed by chlorophyll in chloroplasts and transformed to ATP (adenosine triphosphate) and O_2. However, besides light and CO_2, microalgae also utilize sugars, N, P, and potassium (K) for their growth and convert them into organic molecules (lipids, proteins, and carbohydrates). Unlike photoautotrophic microalgae, heterotrophic microalgae consume organic compounds as a source of energy and carbon. There are also mixotrophic microalgae which can switch their metabolism between photoautotrophic and heterotrophic (Bernnan et al., 2010). Microalgae can decrease greenhouse gas emissions by consuming CO_2 from industrial flue gases (bio-fixation), and they can reduce pollution by removing nutrients (P, N, K, NH_4^+) from wastewater (Patel et al., 2017).

From the current commercial point of view, microalgae are widely used as supplements for human health, animal feed, cosmetics and high-value molecules (fatty acids, pigments, phycobiliproteins) (Spolaore et al., 2006).

4.1 Cultivation conditions and composition of algae

Microalgal growth follows typical microbial growth curve (Figure 3): starting with the lag phase, continuing with exponential, linear and stationary phase and ending with death phase. The growth and metabolism of microalgae vary on the species and cultivation conditions. Optimal pH for the growth of most of the microalgae is between 7-9, and optimal temperature is between 20-30 °C. Some of the species can have optimal cultivation conditions out of the mentioned range (psychrophilic or thermophilic) (Cuellar-Bermudez et al., 2017; Patel et al., 2017). Light also plays a significant role in microalgal growth being the source of energy, and proper light intensity should be provided when growing microalgae. The requirement for essential nutrients (N, P) and trace elements (K, Fe, Mg, S, Ca, Zn, Cu, Mn) depends on microalgal species, but it could be evaluated based on microalgal composition analysis (Jaatinen et al.,2016).

Temperature and pH can affect the nutrient uptake and composition of the microalgae. Microalgal composition, in general, is rich in proteins (30-50% of total organic matter), carbohydrates (20-40% of total organic matter) and lipids (8-15% of total organic matter). Also, microalgae contain amino-acids, fatty acids, pigments, antioxidants, minerals, vitamins but even toxins (Spolaore et al., 2006; Wang et al., 2013). For instance, the temperature can alter the concentration of the unsaturated fatty acids in the microalgal membrane. pH variations affect not only the growth but also the concentration of lipids (lower pH can result in higher lipid content). Increasing salinity of the cultivation media can increase the concentration of monounsaturated fatty acids (Cuellar-Bermudez et al., 2017; Patel et al., 2017). N deprivation leads to lipid accumulation (Wang et al., 2013). The microalgal composition also varies in the growth phase. For example, proteins tend to be predominant in exponential phase, while sugars tend to be predominant in stationary phase (Patel et al., 2017; Trivedi et al., 2015). This opinion was also supported by the study of Wang et al. (2013) where the alteration of light intensity and N supply could achieve accumulation of proteins and carbohydrates in the early growth phase and accumulation of lipids in the later growth phase in microalgae *Scenedsmus dimorphus* (Wang et al., 2013).

The cultivation conditions need to be monitored during the whole cultivation peroid, and the proper mixing of microalgae must be provided because even small alteration of a cultivation condition can cause a different effect on microalgae (Patel et al., 2017). Sometimes changing the cultivation condition and causing stress conditions can be desirable for achieving a specific microalgal product. For example, microalgae *Haematococcus pluvialis* can accumulate high-value pigment astaxanthin by increasing light intensity (Wayama M, 2013).

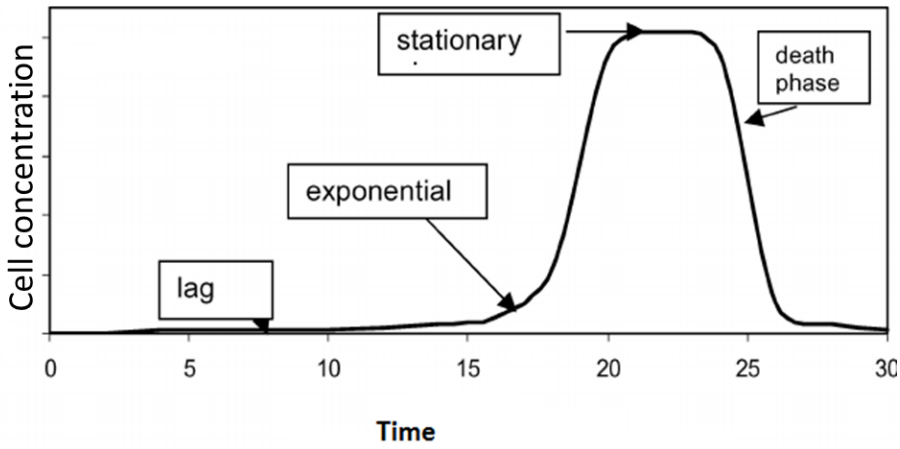

Figure 3. Growth curve representing growth rate of microalgae (Modified from (Tindall et al., 2005)).

4.2 Mechanism of nutrient uptake and microalgal metabolism

Microalgae cannot survive without phosphorus and nitrogen. That is because P and N are involved almost in all vital biochemical processes of the cell.

4.2.1 Metabolic pathway of phosphorus

It is still not clearly explained how the exact mechanism of P uptake works, but it enters the microalgal cell as inorganic phosphate (PO_4^-) (Solovchenko et al., 2016).

PO_4^- does not spontaneously diffuse through the lipid bilayer of the cell membrane due to its negative charge. However, when the environment is rich in PO_4^-, passive diffusion can be preferred. Otherwise, several theories suggest that there are two mechanisms of active transport through plasmalemma: a) assimilation of PO_4^- inside the cell and b) luxury PO_4^- uptake (Schmidt et al., 2016; Solovchenko et al., 2016). The former one transforms PO_4^- into acid-soluble PO_4^-granules and the later one transforms PO_4^- into acid-insoluble granules. Acid soluble PO_4^- granules take part in the metabolism, and as acid insoluble granules they are stored inside the cell till the time when the external PO_4^- is limited (Schmidt et al., 2016).

P uptake is influenced by the level of cell starvation. Phosphate starvation is defined as a reduction of P_{intra} (intracellular P concentration) in microalgae below their normal metabolic needs for P. Cells can actively respond to the external changes of the PO_4^-concentration. In the case of very high P_{extra} (extracellular P concentration) microalgae consume only certain amount of PO_4^- depending on their needs (external conditions, microalgal metabolism) but they will not uptake PO_4^- in excess. Proposed opinion is that high P_{intra} represses metabolic pathways responsible for P uptake from the environment resulting in poor or no P uptake. Consequently, if microalgae starve (due to very low P_{extra}), they

consume P stored inside the cell (acid-insoluble PO_4^- granules) and the P_{intra} drops activating metabolic pathways responsible for P uptake from the environment and rapid accumulation of P in the form of the acid-soluble PO_4^- granules (Azad et al., 1970; Schmidt et al., 2016; Solovchenko et al., 2016).

When it comes to the luxury uptake, excess P is stored inside the microalgal cell in the form of the acid-insoluble PO_4^- granules (Schmidt et al., 2016). There is no need for the previous starvation of microalgae for luxury uptake. Additionally, P is stored inside the microalgal cell even if they can easily obtain it from the environment. Several studies suggest that the luxury uptake is the result of microalgae evolution to survive during the time of nutrient depletion (Solovchenko et al., 2016).

During the PO_4^- uptake, ATP is hydrolyzed, and the membrane potential is changed. The cations (H^+ or Na^+) are involved in the transport along with PO_4^-. In very low concentration of external P, the uptake process is facilitated by releasing bioavailable P with the help of extracellular enzymes (e.g., phosphatase) (Solovchenko et al., 2016). Figure 4 describes movement of PO_4^- outside and inside microalgal cell.

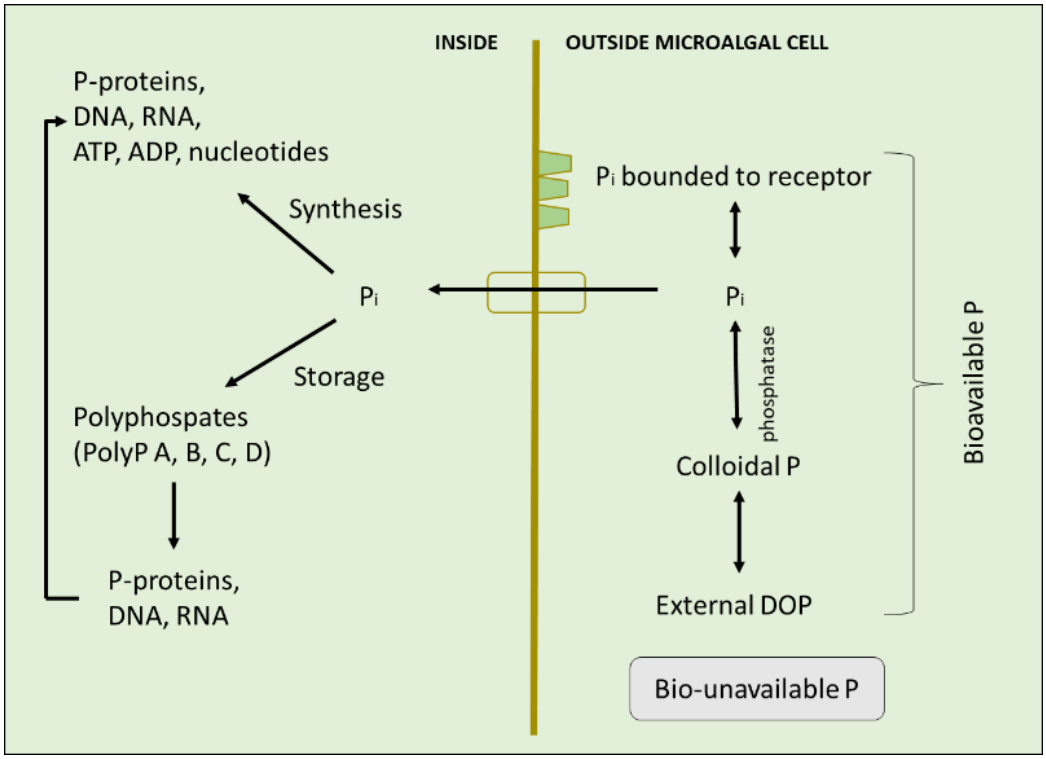

Figure 4. Graphical representation of P flow inside (left side) and outside the microalgal cell (right side). Right side: P_i (inorganic P) can be directly transferred inside the algal cell through active transport or alternatively it can be bounded to the receptor in the membrane. Other forms of P (colloidal P and dissolved organic P; DOP) have to be converted into P_i by extracellular enzymes prior the uptake. The bio-unavailable P is not converted by enzymes. Left side: Transported P_i is used either directly in the synthesis of biomolecules (e.g. DNA, ATP) or it is stored as one of the 4 types of polyphosphates (PolyP A-D) (Modified from (Solovchenko et al., 2016)).

Role of phosphorus inside the microalgal cell

The amount of P inside the microalgae varies between 5-10 mM, but the P uptake is usually less than 4 μM for most of the species. Right after the entry of the P, a large part of it is consumed by metabolic reactions, like phosphorylation and dephosphorylation during protein synthesis. Another part is deposited inside the cell, and it could be used as short-term energy in the form of ATP (adenosine triphosphate). ATP is the main product of photosynthesis. Hence, it plays a crucial role in microalgae. For long-term energy, P could be stored in the form of carbohydrates, lipids or polyphosphates. The polyphosphates take an important part in microalgal metabolism along with ATP. They are stored in vesicles or vacuoles, but their exact biosynthesis and degradation are not known. However, they can also be involved in the formation of ATP (Solovchenko et al., 2016).

Influence of cultivation conditions on phosphorus uptake

The intracellular P concentration (concentration inside the microalgal cell, P_{intra}) or P uptake depend on external factors like temperature, light intensity, extracellular P concentration (concentration in the environment, P_{extra}), microalgal density, mixing and the diurnal cycle. Nevertheless, microalgae require for their growth some minimum P critical concentration (P_{cc}) in culture media regardless the external factors. In other words, microalgal growth is reduced when the P_{extra} is less than P_{cc}. At the same time, P_{cc} changes with changing external factors (Azad and Borchardt, 1970).

Temperature is an important factor that can influence microalgal growth either directly or through the culture media. Direct influence on microalgae affects the speed of different metabolic reactions inside the cell. Increasing temperature and high P_{extra} presents positive effect on P luxury uptake. Decreasing temperature has been shown to increase P_{cc} (Azad and Borchardt, 1970; Powell et al., 2008). A study by Schmidt et al. (2016) concentrates on the cultivation of microalgae and P removal from wastewater in cold climate. However, the results and the algal behavior are uncertain under cold conditions (Schmidt et al., 2016).

Under intensive light irradiance, microalgal growth is fast, P_{extra} is utilized for microalgal metabolism, and thus P_{intra} decreases. A similar phenomenon happens when the P_{extra} is low. Then the biomass turns to carbon-rich biomass (decrease in P luxury uptake) (Powell et al., 2008; Schmidt et al., 2016). High light intensities decrease the microalgal demand for P_{extra} and therefore also P_{cc} decrease (Azad and Borchardt, 1970; Powell et al., 2008).

Cell density is coupled with the light intensity. Considering constant illumination with low microalgal densities means more light for cells, and therefore, the result will be similar to intensive light irradiance described in the previous paragraph. High microalgal density causes insufficient light penetration, increased P_{cc} and decreased P luxury uptake (Azad and Borchardt, 1970).

If growing microalgal biomass does not have continuous artificial light supply, then the light is provided only by day and night cycle (diurnal cycle). The growth is naturally slowed during the dark (night) period and enhanced during the light (day) period. Noteworthily, the P uptake is reduced during the dark period. The P uptake during the diurnal cycle is less efficient than under artificial light supply (Azad and Borchardt, 1970).

Proper mixing can enhance the contact of the microalgae with the nutrients, and it can provide better exposure to the light, both resulting in higher P uptake and growth rate (Azad and Borchardt, 1970).

4.2.2 Metabolic pathway of nitrogen

Nitrogen is one of the key players in the synthesis of organic molecules in the cell (e.g., peptides, enzymes, ADP, ATP, DNA, RNA). It could be assimilated to organic molecules from different inorganic forms like nitric acid (HNO_3), nitrogen (N_2), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and ammonia (NH_3). In detail, all eukaryotic microalgae (excluding prokaryotic cyanobacteria) can assimilate only NO_3^-, NO_2^- and NH_4^+ forms. Figure 5 shows the assimilation of N. The first two steps after passing through the microalgal membrane are the reduction of NO_3^- and NO_2^- by nitrite reductase, NADH (nicotinamide adenine dinucleotide) and Fd (ferredoxin). The reduction results in the formation of NH_4^+, which is consequently integrated into amino acids with the help of glutamate (Glu) and ATP. The NH_4^+ is the most advantageous form of nitrogen for microalgae because it avoids reduction reactions and thus it is not so energetically demanding. Therefore, microalgae tend to consume NO_3^- when NH_4^+ is entirely depleted even though NO_3^- is more stable and more predominant in the wastewaters. On the other hand, NO_3^- stimulates the activity of nitrate reductase what could be essential for microalgae (Cai et al., 2013).

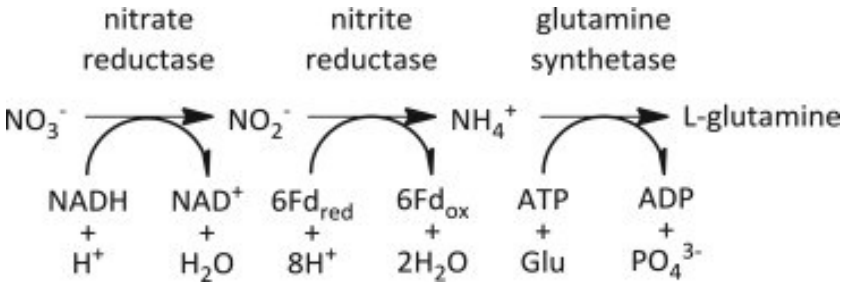

Figure 5. Assimilation reaction of N in microalgal cell (Adapted from (Cai et al., 2013)).

Influence of cultivation conditions on nitrogen uptake

Relatively little is known about the influence of cultivation conditions on N uptake. Some studies show that temperature could alter N uptake in microalgae, but their conclusions are not consistent. For example, the study of Reay et al. (1999) pointed out that there is a temperature difference for NO_3^- and NH_4^+ uptake by microalgae. They demonstrated that nitrogen uptake was efficient in the range of optimal temperature of the specie meanwhile decreasing temperature below the optimum resulted in reduced NO_3^- and NH_4^+ uptake. Moreover, Reay et al. (1999) showed that decreasing temperature has a stronger effect on NO_3^- uptake than on NH_4^+ uptake (Reay et al., 1999). On the other hand, Lomas and Glibert (1999) studied temperature dependence on N uptake for diatoms, and their results showed that with increasing temperature uptake of NO_3^- decreases and uptake of NH_4^+ increases (Lomas and Glibert, 1999). A newer study of Delgadillo-Mirquez et al. (2016) supported the opinion that NH_4^+ uptake is enhanced by elevated temperature but at the same time, NH_4^+ removal could be caused by ammonia stripping. This study also showed that NH_4^+ uptake by microalgae was not detected during dark period (Delgadillo-Mirquez et al., 2016).

Even though there is no clear evidence in the literature that would investigate specifically N uptake by microalgae, it is probable that light intensity, cell density, mixing and microalgal starvation can influence the N uptake similarly like P uptake.

4.2.3 Nitrogen: Phosphorus ratio for nutrient removal

Proper microalgal growth and N and P simultaneous removal from the environment happens if the N: P ratio is in an appropriate range. N: P ratio for freshwater microalgae is between ranges of 8:1 to 45:1 (N: P) and it depends on the metabolic pathways of different microalgal species. Consequently, microalgae can grow in wastewaters that have proper N: P ratio (Cai et al., 2013; Whitton et al., 2016).

4.3 Technologies for microalgae cultivation

Growing microalgae brings not only environmental but also social benefits since microalgae do not compete with food crops for the land. Microalgae withstand even more extreme environment such as arid land or desert. However, despite their immunity against extreme environment, the large-scale cultivation can bring some limitations like the inability of some species to grow in high density and their sensitivity to contamination (Patel et al., 2017). There are several systems to grow microalgae in large scale:

Cultivation in open ponds

Open ponds are artificially made shallow basins where continuous mixing is provided by paddle wheel (raceway pond, RwP). RwP is economical solution for microalgae cultivation because it is easy to construct and maintain them. Open ponds find their place mainly in the wastewater treatment processes where the exposure to the contamination is not the primary concern. Furthermore, they are a good choice when the microalgae consume industrial flue gases as a main source of carbon. The restriction comes with the low homogenization of nutrients inside the RwP caused by insufficient mixing, a requirement for a large land area, difficult control of evaporation and temperature (Patel et al., 2017; Solovchenko et al., 2016).

Cultivation in photobioreactor

The main feature of photobioreactors (PBR) is that they are entirely closed systems where the culture conditions (pH, T, light, CO_2) and protection from contaminants can be managed very well. The most common types include vertical, circular, horizontal, tubular and flat panel PBR. Preference of PBR over the open ponds is predominant in situations like cold climate, high price for land, unstable yield and biomass quality or sensitivity of microalgae to external factors. However, higher operational cost, possible overheating or low oxygen transfer should be taken into the account when designing PBR (Patel et al., 2017; Solovchenko et al., 2016).

Immobilized microalgae and thin layer cultivation systems

The completely new way of microalgae cultivation is to bind them to the rigid support, e.g., alginate gels. By immobilizing microalgae, mixing is avoided. However, easier harvesting, higher cell retention, and water purification are provided. This method promises high values of P uptake (up to 70%) from wastewater by algae and thus, immobilized algae can be almost directly used as bio-fertilizer rich in P. On the other side, issues like limited nutrient diffusion and photoinhibition need to be solved (Solovchenko et al., 2016). Similar to immobilized microalgae, thin layer cultivation is exploiting short light path. Available light can support microalgal density and transfer of CO_2 and O_2 way better than in the case of PBR or open ponds. Moreover, not only density is supported, but also consequent microalgal pumping and harvesting reduce the expenses (Solovchenko et al., 2016).

4.4 Harvesting of microalgae

Harvesting is one of the most crucial steps in the whole microalgal production. Microalgal biomass is dissipated in culture media (0.1-2.0 g of dry weight/l of reactor volume). Hence harvesting recovers and concentrates the microalgal biomass from the culture media. Criteria for selection of harvesting method should take into the account cell properties (size,

density), efficiency of dewatering, toxicity, applicability for industrial scale, time and maintenance effectivity, reuse of culture media and characteristics of the final product (human use or industrial production). The efficiency of selected harvesting method will contribute to the price of final product (20-30% of the total cost) (Al hattab et al., 2015). Table 1 summarizes harvesting methods with main principle of separation, advantages, and drawbacks of individual harvesting methods.

Table 1. Summary of microalgal harvesting methods (Al Hattab et al., 2015; Patel et al., 2017; Chen et al.,2011).

Name	Principle of separation	Advantages	Drawbacks
Sedimentation	- settling of biomass by gravity	- effective concentration of algae - low cost	- low reliability - addition of flocculant agents - long time - additional energy required
Filtration	- biomass retained on the filtration cloth - based on pressure difference (vacuum, pressure, gravity)	-preservation of the cells -cake collection with low moisture -complete removal of cell debris and algae	- membrane replacement or washing to avoid clogging
Centrifugation	- application of centrifugal force - pressure differential for particle separation	- high removal efficiency - high concentration of biomass	- highly moisturized biomass -complex structure - high cost - difficult maintenance
Flotation	- gaseous bubbles forcing the microalgae to float to the surface	- faster and more effective in comparison with sedimentation	- not suitable for large scale - high energy input and operational cost
Chemical flocculation	- inorganic or organic flocculants - neutralization of the charge and particle bridging	- can handle large amount of microalgae - used with wide range of algal species - cost effective	- introduction of chemicals - toxicity of flocculants
Electrolytic coagulation	- generation of electric current and microalgal aggregation	- versatility - energy and cost efficiency - safety and selectivity	- cathode fouling - change in microalgal composition

4.5 Microalgae grown on urine - State of the art

Struvite precipitation and ammonia stripping are the only methods applied for nutrient recovery from urine in large scale so far. Nevertheless, their limitations are high energy inputs and low yields of recovered P and N (Tuantet et al., 2014).

There are only a few studies that investigate growing microalgae in concentrated urine or urine with low dilution factor, and all of them were conducted in small, laboratory scale (Tuantet et al., 2014). Challenge of concentrated urine relies on its partially unknown composition and the hydrolysis of urea into NH_4^+ which is further converted into free NH_3. Microalgae can grow on NH_4^+ but elevated NH_3 concentration could inhibit the growth (toxic concertation could be in the range of 20 – 664 mg/l of NH_3 depending on the microalgal specie) (Tuantet et al., 2014; Collos et al., 2014).

The study of Chang et al. (2013) tested the growth of cyanobacteria *Spirulina platensis* on 120 times diluted human urine (HU) and synthetic urine (SU). The cultivation conditions were: 1.2 l PBR (photobioreactor) with CO_2 supply, light-dark cycle and maintained 30 °C. Moreover, this study investigated mixotrophic conditions in SU by addition of sodium acetate. They were obtained maximal biomass of 0.81 g of dry weight/ l of reactor volume with 96% removal of P and 98% elimination of urea in HU. The composition of biomass was 35.4% of protein/ dry weight and 19.8% of lipids/ dry weight (Chang et al., 2013). The study of Tuantet et al. (2014) reported the highest biomass productivity of 15.4 g/l in 3 times diluted HU. *Chlorella sorokiniana* was cultivated in 1 l PBR, with CO_2 supply and continuously illuminated urine. P and N removals were 76% and 87% respectively. In addition, Tuantet et al. (2014) tested SU with 2-20 dilutions, and the result was 6.0 g of dry weight/l of reactor volume and 2.9 g of dry weight/l of reactor volume for 5 and 20 diluted urine respectively. Biomass grown on SU was rich in proteins (48%) and biomass grown on HU was rich in lipids (25%) (Tuantet et al., 2014). Another study of Tuantet et al. (2013) tested the growth of *Chlorella sorokiniana* on diluted (5x,10x) and concentrated (0x, 2x) HU with the addition of trace elements (TE), continuous illumination, CO_2 supply and incubated in batch microtiter plates at 30 °C. They achieved maximal microalgal growth on 20x diluted urine with TE and highest growth rates on 5x and 10x diluted HU. Interestingly in this study, the composition of HU obtained from females and males was tested, but the analysis showed that there was no difference between HU from females and males (Tuantet et al., 2014). Jaatinen et al. (2016) studied *Chlorella vulgaris* cultivated in 100x diluted HU in 1 l Erlenmeyer flask under continuous illumination and previously adjusted pH. After 21 days of cultivation, 74% of total nitrogen and 80% of total phosphorus were removed, and the biomass concentration was 0.6 g of VSS (volatile suspended solids)/l of reactor volume (Jaatinen et al., 2016). Copens et al. (2016) studied the growth of *Arthrospira platensis* on 20% nitrified urine in 0.8 l submerged membrane PBR. System was operated as a batch with continuous illumination and adjusted pH at 28 °C. The results were 10% N removal, 9.1% P removal and protein content

of 62.4% of dry weight (Coppens et al., 2016). Table 2 summarizes the results obtained from different studies that could be compared to the results obtained in this study.

From the presented studies it can be concluded that the research dedicated to microalgal cultivation on real human urine was focusing mainly on the testing different set up in laboratory scale. Almost all studies used highly diluted urine and provided additional optimal conditions favoring microalgal growth.

One of the ways to close the cycle of circular economy and nutrient recycling is the further use of microalgal biomass. Microalgae grown on urine or another type of wastewater are not suitable for direct human use, but they are suggested to be used as an animal feed, aquaculture feed, fertilizer or source of biofuel (Zhang et al., 2014).

Microalgae could be directly used as a live feed for bivalve molluscs, abalones, zooplanktons and crustaceans. In particular species like *Scenedesmus* and *Chlorella* could be used for the feeding of Artemia and rotifer *Brachionus plicatilis.* Microalgae are easy to ingest, and they are free of pathogens and toxic substances. Suitable biochemical and nutrient content is important for fish health, especially fatty acids in the microalgae (Patel et al., 2017).

Alternative methods for replacing chemical fertilizers suggest using dried dead microalgae as a bio-fertilizer. Despite vigorous effort to use microalgae in agriculture, there is a limited number of studies that are focusing on this topic. One of them is the study Dineshkumar et al. (2017) that evaluates the effect of *Chlorella vulgaris* and *Spirulina platensis* as a bio-fertilizer used for rice farming. Microalgae are source full of N, and they do not cause soil and water pollution. Both microalgae enhanced overall rice condition (improved rice height, number of leaves and leave are per plant). They reduced additional consumption of chemical fertilizer up to 75%, increased crop yields up to 20% and improved soil biological and chemical properties (Dineshkumar et al., 2017).

Table 2. Summary of results of microalgae cultivation with urine obtained from different studies; (HU- human urine, PBR- photobioreactor, NT-not tested, DW- dry weight, VSS – volatile suspended solids).

Algal strain	Urine specification and dilution	Culture condition	Biomass yield per liter of reactor volume	P_{tot} removal (%)	N_{tot} removal (%)	Reference
Chlorella vulgaris	1:100 not sterilized HU	Batch cultivation in 1l Erlenmayer flask addition of trace elements	0.6 g of VSS	80	74	(Jaatinen, S., Lakaniemi, A.M, Rintala, J., 2016)
Chlorella sorokiniana	1:3 HU	Batch flat panel PBR, 1l CO_2, Mg, Fe supply N: P ratio optimization	15.4 g of DW	76	87	(Tuantet et al., 2014)
Spirulina platensis	1:120 HU	Batch PBR, 1.2 l Addition of sodium acetate CO_2 supply	0.81 g of DW	96.5	NT	(Chang et al., 2013)
Scenedesmus acuminatus	1:50 fresh HU	Semi-batch tank, 130 l Mg, Fe supply	0.16 g of DW	36	67	(Adamsson, 2000)

5. MATERIALS AND METHODS

The experiment included three major tasks: (a) culturing of *Scenedesmus acuminatus* in controlled conditions for future use as inoculum; (b) screening different dilutions of source-separated urine and their impact on *S. acuminatus* growth; and (c) cultivation of *S. acuminatus* on diluted urine in raceway ponds. This chapter describes the methodology followed to achieve these specific tasks in detail.

5.1 Culturing of *Scenedesmus acuminatus*

Scenedesmus acuminatus was obtained from the Culture Collection of Algae (SAG) at the University of Göttingen, Germany. A 1 l glass photobioreactor (PBR) (Figure 6) with working volume of 700 ml at ~22°C was used to culture the microalgae. The PBR was inoculated with 5 ml of inoculum, and it was under continuous illumination of the fluorescent lamp (Osram L 18W/965 Biolux, Germany) of the intensity of 40 µmol photons m^{-2} s^{-1}. *S. acuminatus* was grown in N8 media with the composition (per liter): 0.5055 g KNO_3; 0.74 g KH_2PO_4; 0.2598 g Na_2HPO_4; 0.05 g $MgSO_4$ x $7H_2O$; 0.0175 g $CaCl_2$ x $2H_2O$; 0.0115 g FeNaEDTA x $3H_2O$; 0.0032 g $ZnSO_4$ x $7H_2O$; 0.013 g $MnCl_2$ x $4H_2O$; 0.0183 g $CuSO_4$ x $5H_2O$ and 0.007 g $Al_2(SO_4)_3$ x $18H_2O$. The pH was adjusted to 8.5 with 1 M NaOH, and prepared media was filtered with vacuum filter using 45 µm Millipore filter. The culture stock was mixed by purging with air. Carbon dioxide (CO_2) was purged in the media when faster growth was required. Air and CO_2 were dispersed by glass distribution tube (porosity 0, diameter 22 mm, Duran Group, Germany) from the bottom of the PBR. The airflow was controlled by the flow meter (Dwyer Instruments Inc., USA) at a constant flow rate of 0.2 l/min for air and 20 ml/min for CO_2. The distilled water was added to replace the water loss caused by evaporation. The initial optical density (OD_{660}) of the culture at 660 nm was ~0.5. When the OD_{660} reached the value ~5.5 – 5.8 (growth saturation), 5 ml of washed stock cultures were inoculated into 700 ml of fresh media and the saturated culture was transferred into 5 l canisters and stored in the dark at 8°C (used as inoculum in further studies). Storing microalgae in cold room will inactivate their biological functions and inhibit their reproduction, but it will not destroy them. OD_{660} and pH values of growing stock culture were measured daily.

Figure 6. Photobioreactor with *S.acuminatus* grown in N8 media.

5.2 Screening of different dilutions of urine as growth media

Source separated urine (later mentioned as urine) was collected from Hiedanranta, Finland by using urine diversion toilets and stored for at least 6 months in Hiedanranta before use to destroy potential pathogens (Maurer et al., 2006). Urine was stored at an ambient temperature (5-20 °C) in 2000 l plastic tanks.

The possibility of using source-separated human urine as growth media for *S. accuminatus* was tested in laboratory experiments. Urine was obtained from Hiedanranta storage (Table 3) and stored at 8 ºC for two months before diluting it with tap water. Studied urine dilutions were 0x, 2x, 3x, 4x, 5x, 10x, 15x, 20x and 25x with total liquid volume of 100ml.

S. acuminatus was cultivated in 100 ml of diluted urine in 250 ml Erlenmeyer flask for one week. The volume of the inoculum was 5 ml, and the Erlenmeyer flask was incubated in the shaking incubator (ORBITRON, Infors HT, Switzerland) at 25 ºC at 150 rpm and under continuous illumination with white fluorescent lamps at 40 µmol m^{-2} s^{-1}. Each set up was operated in duplicates (Figure 7). Each day, 5 ml of sample were collected for measurement of OD_{660} and pH.

Table 3. Summary of average composition of urine with standard deviation (n=4 nutrient analysis measurements) used in this study and reference values reported in the literature. All values are in mg/l where appropriate (n.d. not detected; n.r. not reported).

Parameter	Reference values[a]	Urine composition[b]
pH	4.6-8.0	8.8
COD	8000-10000	5500 ± 200
N_{tot}	8000-10000	3480 ± 130
NH_4^+-N	2500-8100	1800 ± 750
P_{tot}	700-2000	190 ± 52
Na	n.r.	690 ± 30
K	n.r.	600 ± 38
Mg	n.r.	n.d.
Ca	n.r.	14 ± 5

[a] Reference values of undiluted urine reported in the literature (Chang et al.2013).

[b] Composition of undiluted urine used in this study.

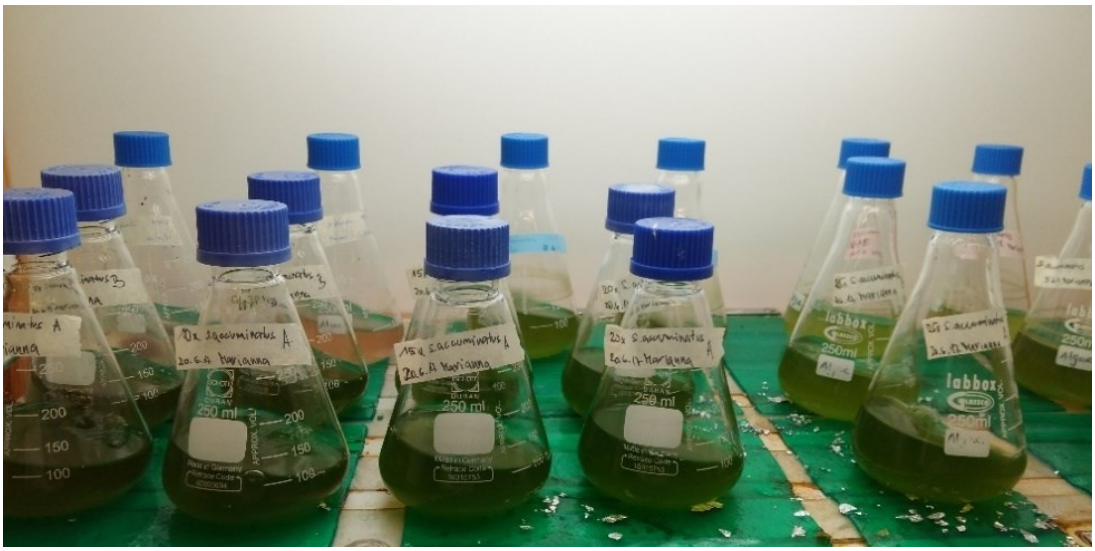

Figure 7. Urine dilution test in 250 ml Erlenmeyer flasks.

5.3 Cultivation in raceway ponds

5.3.1 Hiedanranta industrial area

The microalgal ponds were built in Tampere city district, Finland, called Hiedanranta. Hiedanranta (Figure 8) is an old industrial site that is surrounded by Lielahdenkatu street, Näsijärvi lake, and main railway line. The central development vision is to convert this former industrial area into new urban construction that will be based on principles of circular economy. Namely, the effort of economically and environmentally sustainable development is focused on building low-carbon urban environment; spaces combining housing, leisure time, work and services; appropriate transport system; diverse utilization of urban greenery and food and energy self-sufficiency (Lehtovuori et al., 2016).

Figure 8. Hiedanranta area, Tampere, Finland (tampere, 2017).

5.3.2 Greenhouse plan

The pilot raceway ponds used for the study were built inside a greenhouse in Hiedanranta area. Greenhouse (Figure 9) was constructed from preserved wood and 10 mm transparent polycarbonate cell structure sheet. Figure 10 shows the graphical representation of greenhouse plan: the dimensions of the greenhouse are 16.5 m x 7.2 m and it had two entrances.

Figure 9. Greenhouse with microalgal ponds.

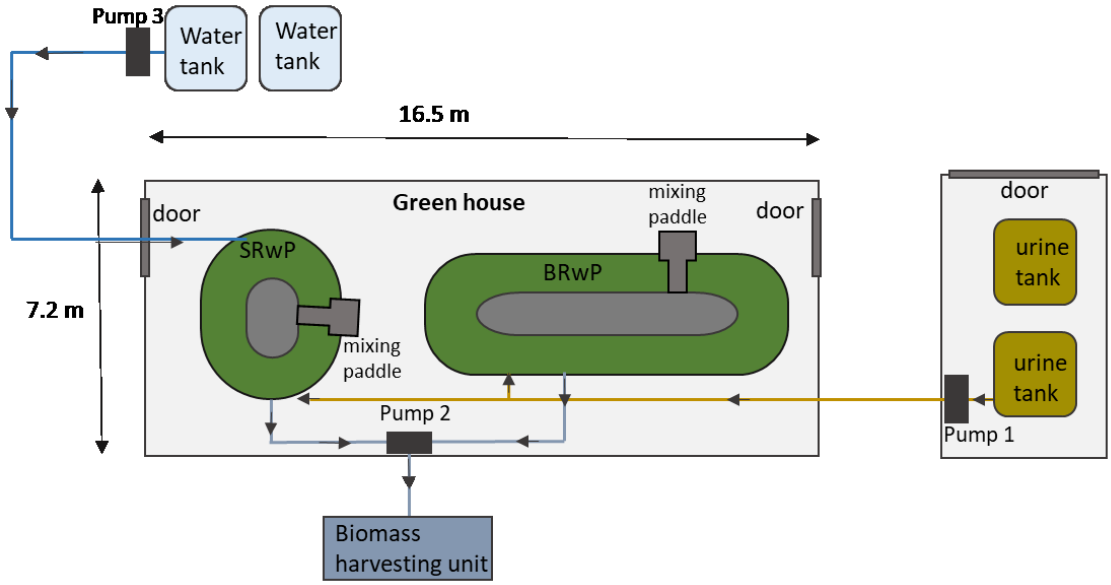

Figure 10. Detailed greenhouse plan.

The raceway ponds (RwP) were shaped on gravel and sand and covered by 1,5 mm thick high-density polyethylene sheet. The dimensions of two RwPs were: a) length 3 m, channel width 0.5 m, depth 0.4 m; b) length 7 m, channel width 1 m, depth 0.6 m. RwPs were equipped with one paddle wheel constructed from 4 mm polypropylene sheet and stainless-steel core (Figure 11). Paddle wheel was continuously rotated with electric gear motors (230/400 V, 50 Hz, 850 rpm, Regal Beloit, Marathon motors, USA). The size of the paddle wheel was adjusted according to the actual depth and width of the ponds enabling constant unidirectional mixing of the culture. The speed of the paddles was controlled by the controlling panel (Figure 11).

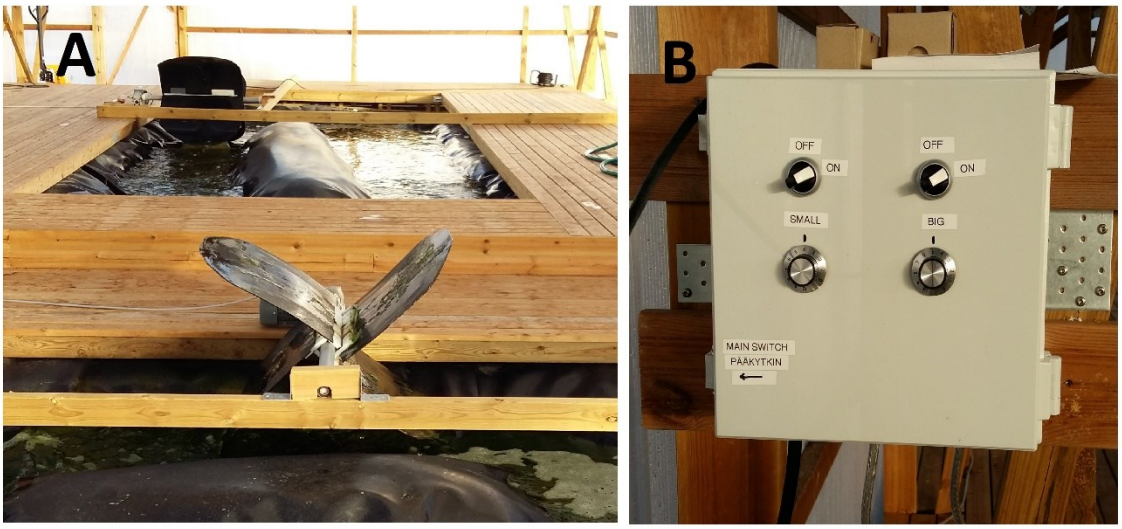

Figure 11. A) paddle wheel; B) controlling panel of the paddle wheel speed. Upper knobs serve for turning on/off of the paddle wheels and lower knobs serve for adjusting the speed of the paddle wheels.

5.3.3 Water and urine supply

Urine was obtained from nearby urine collection facility (Hiedanranta, Finland) and it was stored in 2000 l tanks (Figure 12). The tanks were placed inside a shipping container beside the greenhouse (Figure 10). Furthermore, the urine was pumped out of the tank with a submersible pump (XK 8003, ESPINA-IKH, Finland). For concentrated urine nutrient analysis, 2x 500 ml of urine were collected at the beginning of experiment from urine collection tank and stored at 4 °C in the refrigerator.

Tap water was provided by Tampere city, and it was stored in 2000 l tanks beside the greenhouse (Figure 10). The water was pumped out of the tank with two submersible pumps.

Figure 12. Source separated urine collection facility and storing tanks.

5.3.4 Batch raceway pond operation

Smaller raceway pond was operated as a batch system with working volume of 400 l. The urine was added only at the beginning of the cultivation process and rest of the time just tap water was added to replace evaporated water. *S. acuminatus* grown in the laboratory PBR and stored at 8 °C was used as inoculum. 10 l of *S. acuminatus* with approximate optical density 0.5 was used to inoculate the RwP which was operated with 20x diluted urine. The time for filling the pond with urine and water was calculated based on technical parameters of the pumps. The pond contents were continuously mixed with paddle wheel with a constant velocity of 10 rpm. The first inoculation of the smaller RwP with *S. acuminatus* (10 l of culture with OD 0.5) was prior the batch experiment. *S. acuminatus* was left to grow for 10 days. On the 10th day, around 350 l of the grown culture was used as inoculum (OD 0.75) for semi-continuous RwP, and rest of the culture (50 l) was used as inoculum for batch operation in the smaller pond. Daily routine included brushing the microalgal biomass settled on the pond´s shore, taking the samples (250 ml), measuring the light intensity and temperature. Samples were collected in morning hours (9-11 a.m.) and analyzed in the laboratory by measuring OD, pH and nutrient analysis (P_{tot}, N_{tot}, NH_4^+-N, cation IC). The daily ambient temperature values were obtained from Finnish Meteorological Institute.

5.3.5 Semi-continuous raceway pond operation

Bigger raceway pond with working volume 2000 l was operated initially as a batch system for 10 days and after that as a semi-continuous system. Microalgae from the batch RwP were used as inoculum. Approximately 350 l of the inoculum with OD_{660} 0.75 were pumped from the batch RwP (after 10 days of growth) to the semi-continuous RwP and mixed with 100 l of urine and 1550 l of water. When the microalgal biomass was well grown (OD_{660} ~0.5), the batch operation was switched to semi-continuous operation. Dilution used for semi-continuous RwP was 20x at the beginning for 29 days, followed by 15x dilution for 58 days. The time required for feeding the pond with urine and water was calculated based on technical parameters of the pumps. Microalgal culture was continuously mixed with paddle wheel with the constant rotational velocity of 13 rpm. Daily routine included brushing the microalgal biomass settled on the pond´s shore, taking the samples, measuring the light intensity and temperature. Samples were collected in morning hours (9-11 a.m.) and analyzed in the laboratory by measuring OD, pH and nutrient analysis (P_{tot}, N_{tot}, NH_4^+-N, cation IC). The daily ambient temperature values were obtained from Finnish Meteorological Institute.

Microalgal biomass was harvested twice a week by pumping 500 L of microalgal biomass from the pond to a drainage pit (see chapter 5.3.6). Consequently, the pond was refilled with 500 L of tap water and urine according to the operating dilution (20x, 15x). Hydraulic retention time of the pond was maintained at 14 days.

5.3.6 Harvesting and microalgal biomass storing

For the biomass harvesting, the 500 l of microalgal culture were pumped out of the semi-continuous pond and collected in a drainage pit with an attached nylon filter cloth (pore size < 10 μm) (Figure 13) outside of the greenhouse (Figure 10). The time required for filling the drainage pit with microalgal culture was calculated based on technical parameters of the pump. Effluent (filtrated liquid leaking from drainage pit during harvesting) was collected (250 ml) for further analysis in the laboratory. Rest of the effluent was drained out of the pit. The thick and dense biomass was captured on the filter cloth, scrubbed with a spatula and collected in 500 ml plastic bottles (3-4 bottles/ harvesting). Collected biomass was centrifuged for 2 min at 4000 rpm, at 20°C (Sigma 4K15, Germany). Settled algal pellets were washed with deionized water, again centrifuged (2 min, 4000 rpm, 20 °C) and freeze-dried for at least 17 h (CHRIST, Alpha 1-4 LD, Germany) before storing at -20 °C.

Figure 13. Drainage pit for microalgal harvesting.

5.4 Analytical methods and calculations

The OD, pH and DO analysis were done each day (5x/ week), and analysis of the nutrients (P_{tot}, N_{tot}, NH_4^+-N, cation IC) was done twice a week at the day of harvesting from semi-continuous RwP.

The pH and dissolved oxygen (DO) were measured using pH electrode and DO probe (HACH Lange, HQ40D, U.S). Sample (3 ml) from PBR and both the RwPs were collected to track microalgal growth. The growth was monitored by measuring optical density (OD_{600}) at 660 nm using UV-VIS spectrophotometer (Shimadzu UV-1700 PharmaSpec). Additional monitoring of algal growth and health was tracked by bright-field microscope (Axio-Imager, Carl Zeiss, Germany) and photographed using an AxioCam HRc CCD camera equipped with AxioVision software (Carl Zeiss, Germany) (Figure 14).

Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to the Standard Methods (APHA, 1998). The volume of 100 ml from SRwP, BRwP and effluent was filtered through glass fiber filter paper of pore size 1.6 µm (Whatman GF/A).

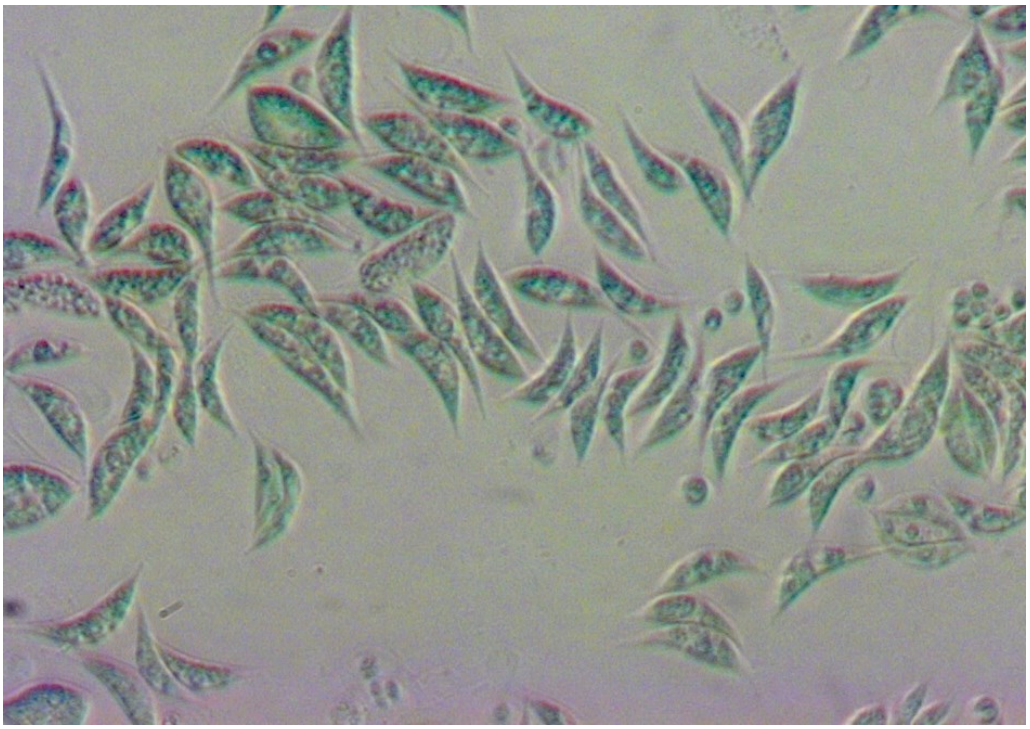

Figure 14. Microscopic picture of *S.acuminatus* growing in N8 media.

Total phosphorus (P_{tot}), total nitrogen (N_{tot}) and ammonia nitrogen (NH_4^+-N)

Total phosphorus (P_{tot}), total nitrogen (N_{tot}) and ammonia nitrogen (NH_4^+-N) were determined with the HACH Lange kit (LCK 350, LCK 338, LCK 303, LCK 304) according to the protocol provided by the manufacturer (HACH, 2017). The suitable concentration range of the kit was based on the approximate calculation of the concentration of the phosphorus and nitrogen in the samples. The samples were heated by thermo-reactor block (HACH Lange, LT 200, U.S) and absorbance was measured with spectrophotometer (HACH Lange, DR 2800, U.S.).

Cation analysis: Na^+, K^+, Ca^{2+} and Mg^{2+}

The cation chromatography (Dionex DX-120) was used to measure the concentration of the cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) in the samples. The samples were prepared according to following procedure: 20 µl of 1 M HNO_3 was added to 20 ml of the sample and mixed well. After that, 5 ml of the prepared mixture was filtered through 0.45 µm PTFE filter (CHROMAFIL Xtra PET 45125, Machinery Nagel, Germany) and used for analysis according to the protocol provided by the manufacturer. As a separating column, IonPac CS12A cation exchange was used with ASRS-300 suppressor (4 mm). Methanesulfonic acid was used as eluent. Analysis of the samples was done according to the ion chromatography standard SFS-EN ISO 10304–1: en.

Chemical oxygen demand (COD)

COD was analyzed according to the Finnish Standard SFS 5504. Total COD (TCOD) was analyzed from urine, and soluble COD (SCOD) was analyzed from the effluent. Effluent was centrifuged (15 min, 4000 rpm) and filtrated (0.45 µm PTFE filter).

Harvesting efficiency calculation

The calculation of microalgal biomass harvesting efficiency was based on the equation:

$$Efficiency = \frac{VSS_{inlet} - VSS_{outlet}}{VSS_{inlet}} * 100\% \quad (1)$$

where VSS_{inlet} is the concentration of volatile suspended solids calculated from semi-continuous pond and VSS_{outlet} is the concentration of volatile suspended solids calculated from effluent of the drainage pit.

6. RESULTS

6.1 Culturing of *Scenedesmus acuminatus*

The normal growth curve and pH change with *S. acuminatus* in growth medium are shown in Figure 15. The highest obtained OD (growth saturation) was 6.4 in 8 days. After that, OD was decreasing signalizing microalgal death. Consequently, average period for one batch cultivation was around 7-9 days and maximum OD, when the microalgal culture was collected, was approximately 5.8. In detail, the period for batch cultivation depended on the initial OD what was verified by measuring OD each day. In other words, the higher the initial OD, the shorter batch cultivation period. Each batch cultivation started at a pH 8.5, and it was fluctuating between 8.5 to 11 during the period of cultivation.

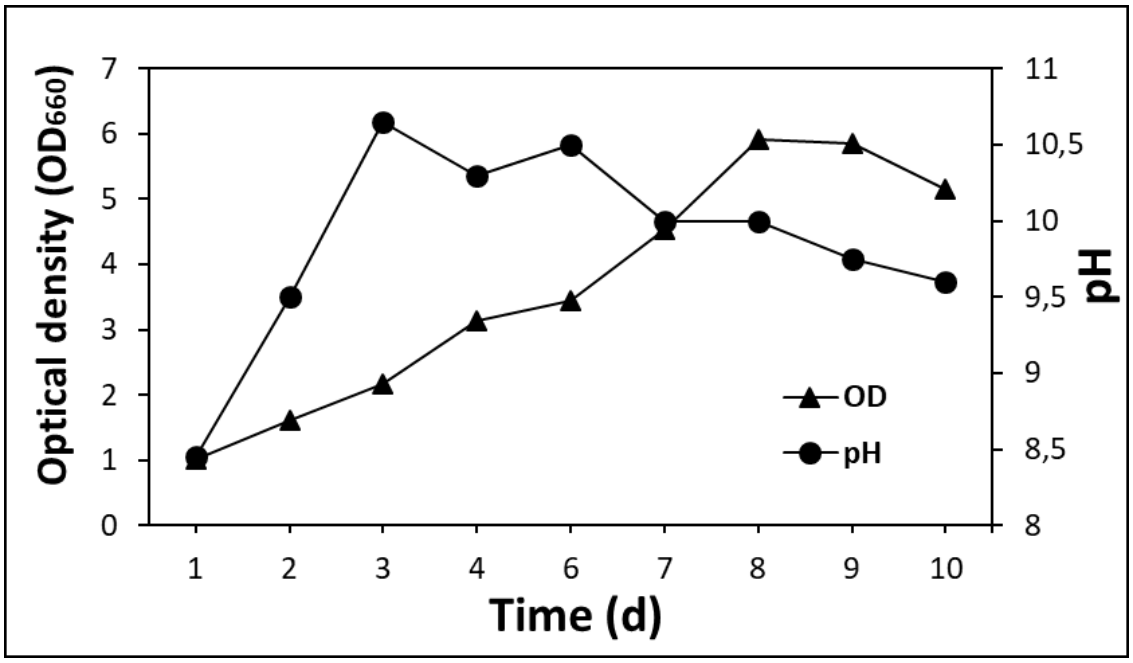

Figure 15. *S. acuminatus* saturation growth curve with maximum OD and pH change.

6.2 Screening of different dilutions of urine as growth medium

Suitability of concentrated urine (referred as 0x), lower dilutions (2x- 5x) and higher dilutions (10x, 15x, 20x, 25x) as a growth medium for S. acuminatus was tested. The first series of dilutions (0x – 5x) did not support microalgal growth. During first 4 days, microalgae were surviving but not growing (Figure 16), except for 5x dilution where slight growth was observed (up to OD 1.0). Nevertheless, after 5th day microalgae were dying regardless of the dilution. pH of medium with lower dilutions increased from 8.8 to 9.2 (Figure 16).

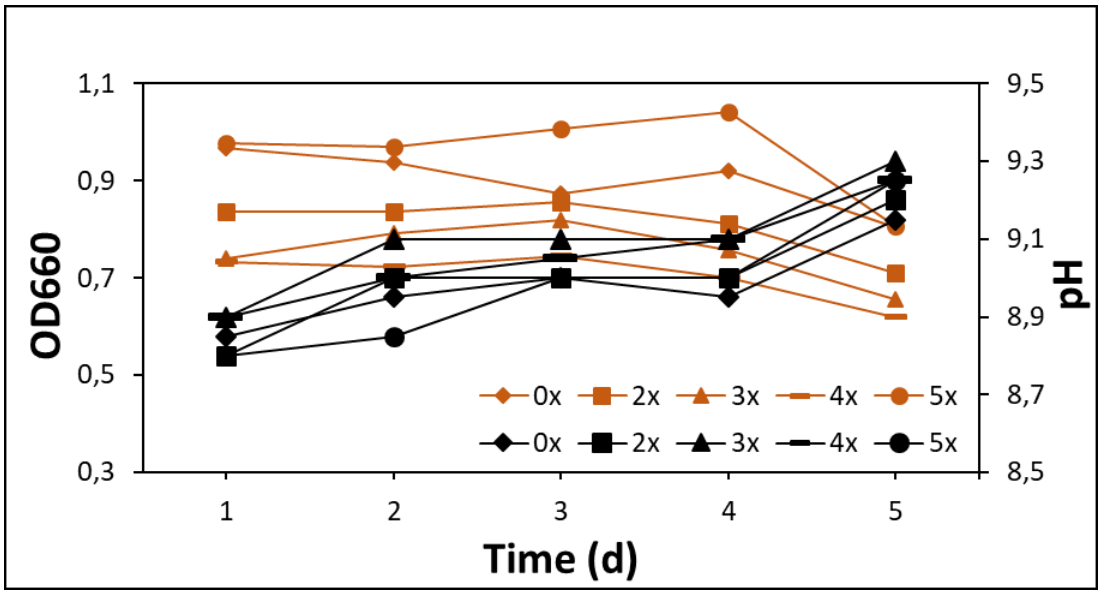

Figure 16. Orange: OD representing *S. acuminatus* growth in concentrated urine (0x) and lower urine dilutions (2x, 3x, 4x and 5x); Black: pH during *S. acuminatus* growth in concentrated urine (0x) and lower urine dilutions (2x, 3x, 4x and 5x).

The second series of dilutions (10x-25x) showed improvement in microalgal growth. Microalgae were growing during first 3 days with the increasing pH, but from 4th day onward they started to die (Figure 17). The dilution that showed continuous growth was only 20x dilution where OD constantly increased from 0.9 to 1.4 which was the highest OD obtained throughout entire screening of dilutions. Despite positive effect of 20x dilution on microalgal growth, the microscopy of *S. acuminatus* growing in 20x diluted urine did not show cells in good shape and condition. Microalgal cells were dispersed and in majority grew individually, rather than forming colonies of 4 (a sign of good shape and condition of the *S. acuminatus* cell) (Figure 18).

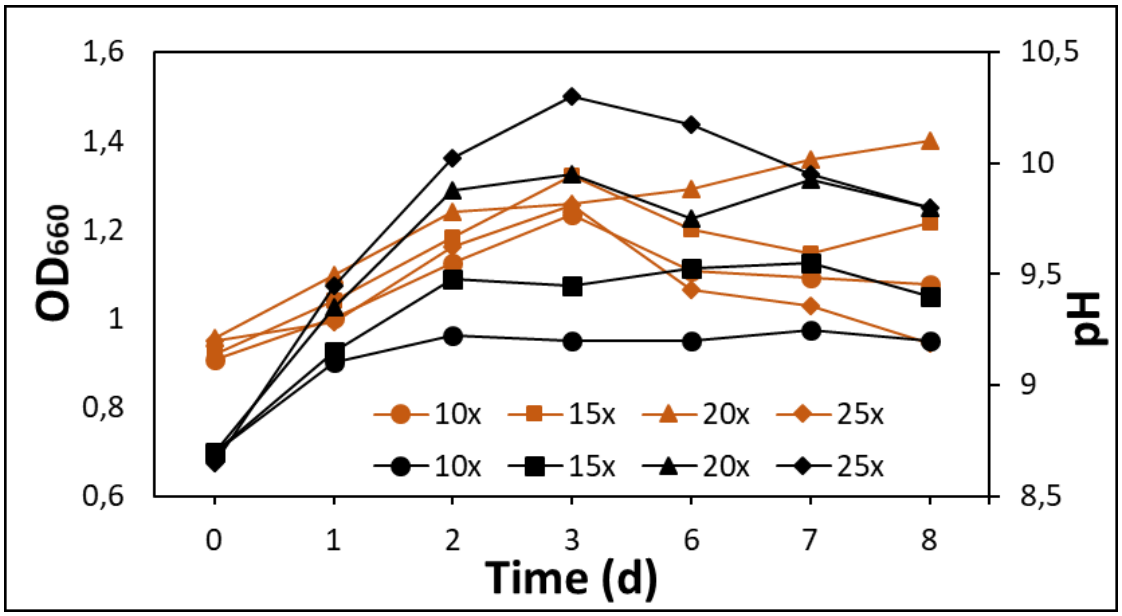

Figure 17. Orange: OD representing *S. acuminatus* growth in higher urine dilutions (10x, 15x, 20x and 25x); Black: pH during *S. acuminatus* growth in higher urine dilutions (2x, 3x, 4x and 5x).

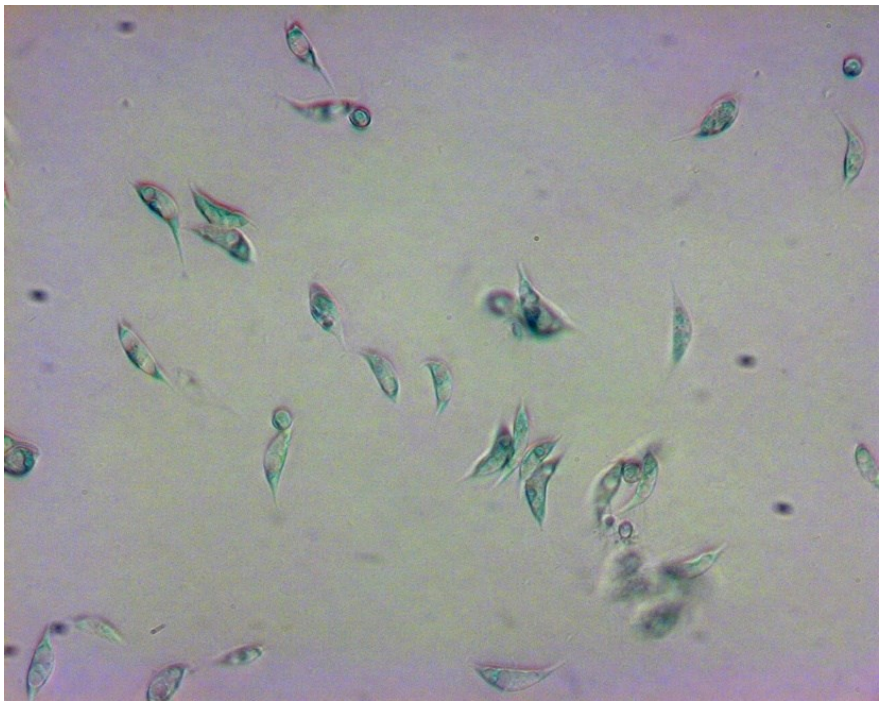

Figure 18. Microscopic picture of *S. acuminatus* growing in 20x diluted urine in Erlenmayer flask.

6.3 Cultivation in raceway ponds

6.3.1 Batch raceway pond cultivation

Batch cultivation was operated in a smaller pond with 20x urine dilution for 94 days, from the middle of July to the middle of October. Figure 19 shows the growth curve from the beginning to the end of the batch cultivation. The growth of S. *acuminatus* was continually increasing for first 40 days when it reached saturation (OD 6.8 and 2.3 g VSS/l of pond volume). After first 40 days of operation, average daily temperature (outside the greenhouse) started to drop from ~ 20 °C below 15 °C, and values of OD and VSS dropped to 5.0 and 2 g VSS/l of pond volume, respectively and stayed constant until the end of the cultivation. Figure 20 shows the relation between microalgal growth and daily ambient temperature. High daily ambient temperatures (15-20°C) were characteristic for the beginning of the cultivation, and with the natural drop of temperature to <14 °C also the biomass growth stopped. The relation between pH and OD is represented in Figure 21. There was a significant fluctuation between individual pH measurements ranging from 7.8 to almost 11. Microscopy (Figure 22) on 14th day revealed that the cells of *S.acuminatus* were in good shape and condition, growing in dense colonies of 4 cells. The nutrient analysis showed that initial concentration of P and N, which was 9.5 mg of P/ l and 173 mg of N /l for 20x diluted non-filtered urine, dropped to 8.6 mg of P/ l and 38 mg of N/ l for non-filtered pond sample in 15 days (in this case, also the same samples were filtered and analyzed to obtain actual value of soluble P in the pond and the concentrations of P and N after 15 days were 0.4 mg/ l and 5.8 mg/ l respectively).

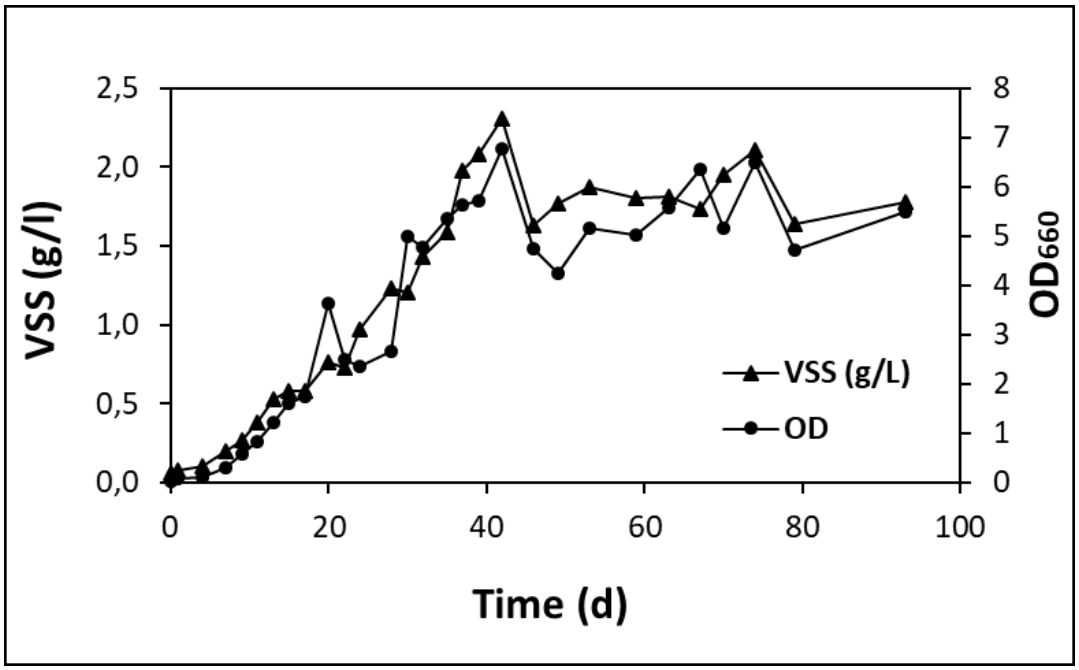

Figure 19. OD and VSS in cultivation of S. acuminatus in 20x diluted urine.

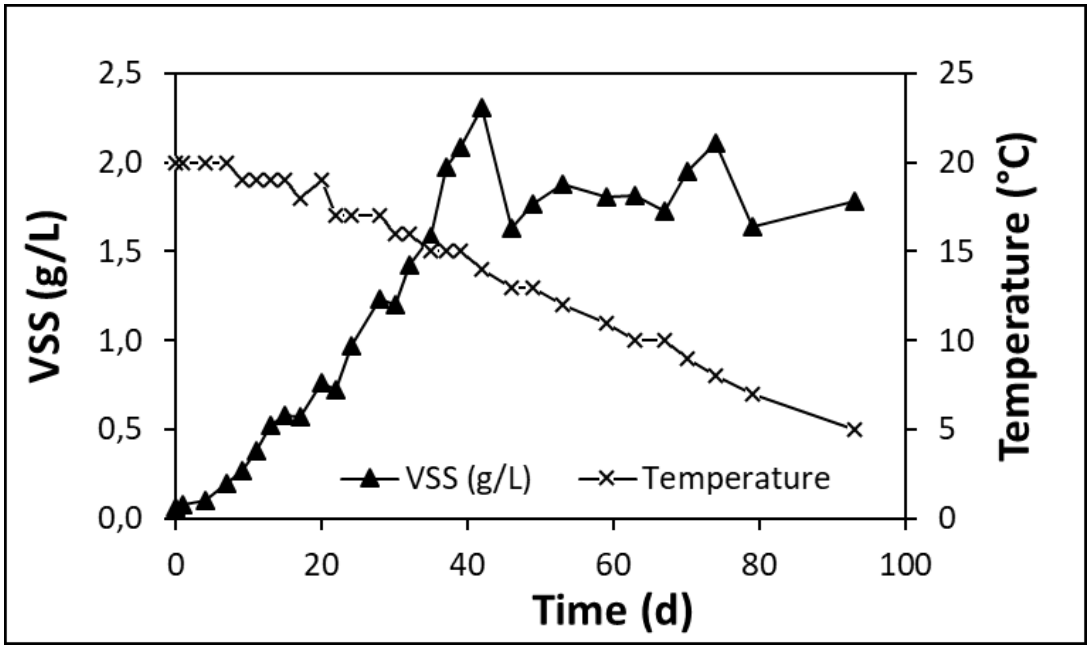

Figure 20. VSS and daily temperature during S. acuminatus growth.

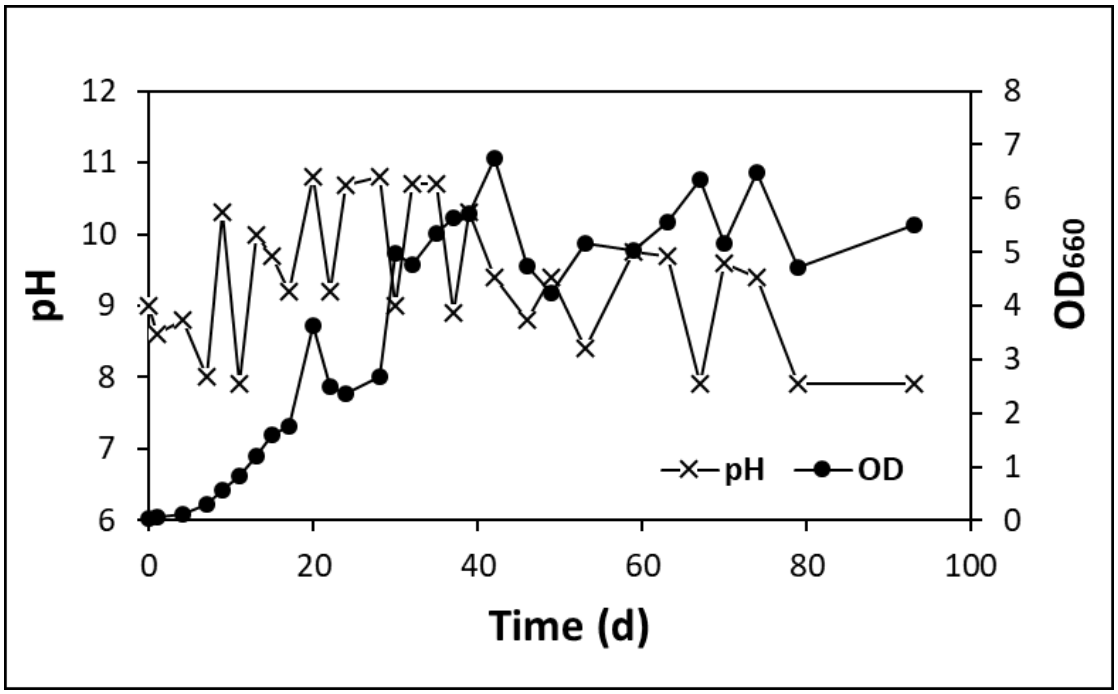

Figure 21. S. acuminatus growth and cultivation pH in 20x diluted urine.

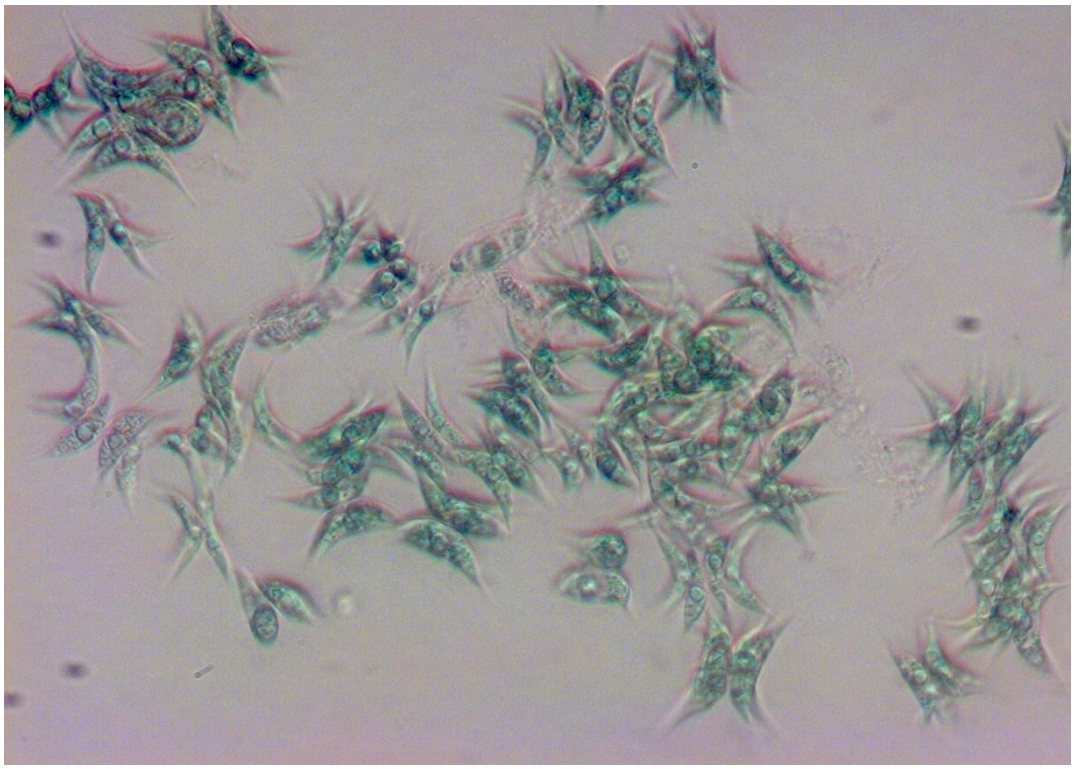

Figure 22. Microscopic picture of S. acuminatus growing in 20x diluted. The picture is taken on day 14.

6.3.2 Semi- continuous raceway pond cultivation

S. acuminatus was cultivated in a semi-continuously operated raceway pond and fed with urine with a dilution of 20x for 30 days (from the middle of July to the middle of August). After 30 days the dilution was changed to 15x until end of the cultivation (64 days, from the middle of August to the middle of October). To keep the semi-continuous mode of the raceway pond, the culture was harvested two times per week.

Figure 23 represents microalgal growth in both dilutions and their relation to the pH. Higher dilution (20x) supported continuous microalgal growth. The maximum OD at 20x dilution of urine after 28 days was 1.14 and pH fluctuation was between 8.5 and 11. After 29 days the medium dilution was decreased to 15x which caused a microalgal reduction from 0.45 g of VSS/l of pond volume to 0.3 g of VSS/l of pond volume (Figure 24). Consequently, the pond was fed only with water for 2 weeks (36th day until 49th day) leaving enough time for microalgal recuperation. When the microalgal culture was recovered, highest OD for 15x dilution reached 0.8. pH was stable between values 8.2 and 8.7 (Figure 23). Figure 24 illustrates the reduction of VSS and daily temperature (outside the greenhouse) during the cultivation.

Harvesting efficiency was calculated as the ratio of VSS collected from the effluent vs. the VSS in the pond. The harvesting efficiency varied between 40 % and 70 % considering only reduction of VSS concentration (Figure 25).

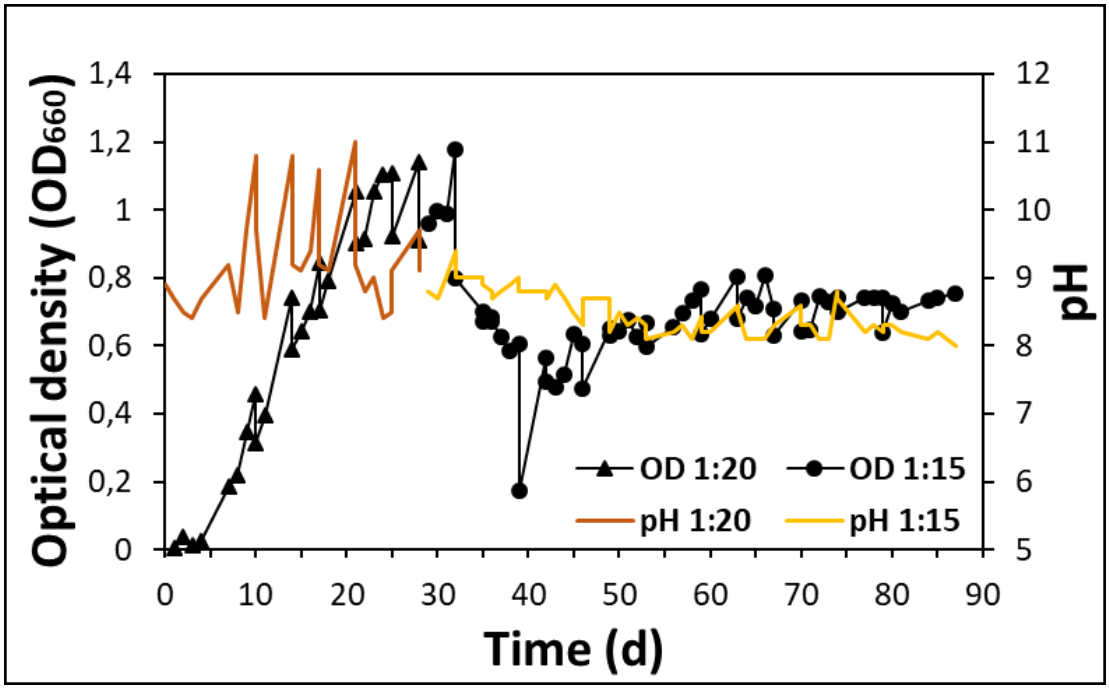

Figure 23. S. acuminatus growth curve in 15x and 20x dilutions with medium pH.

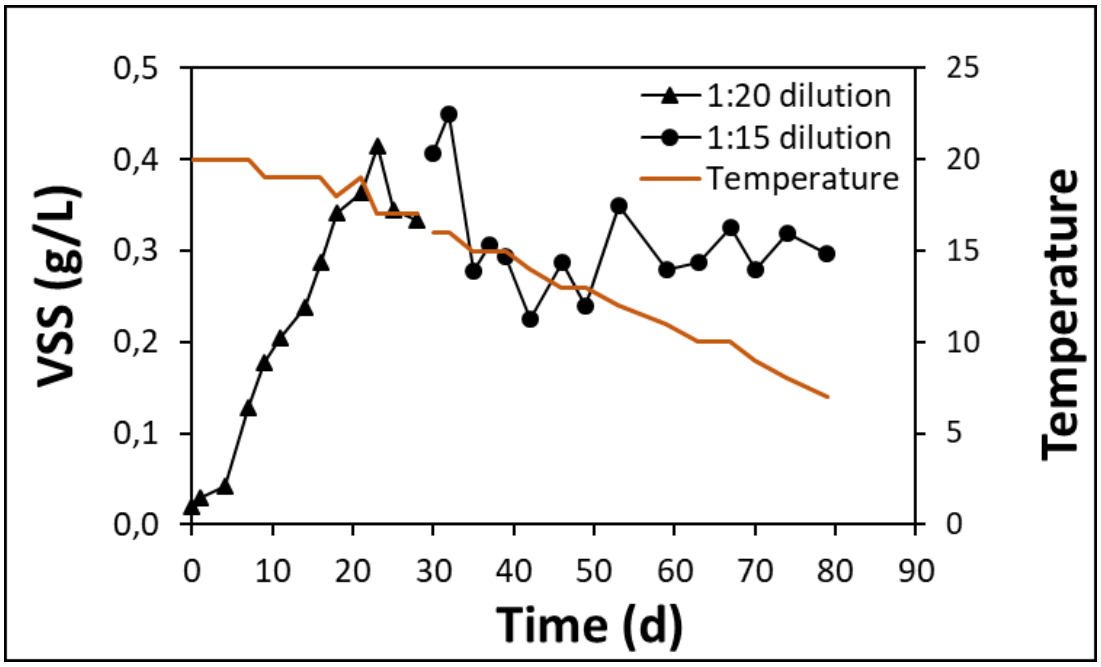

Figure 24. VSS and temperature during S. acuminatus growth.

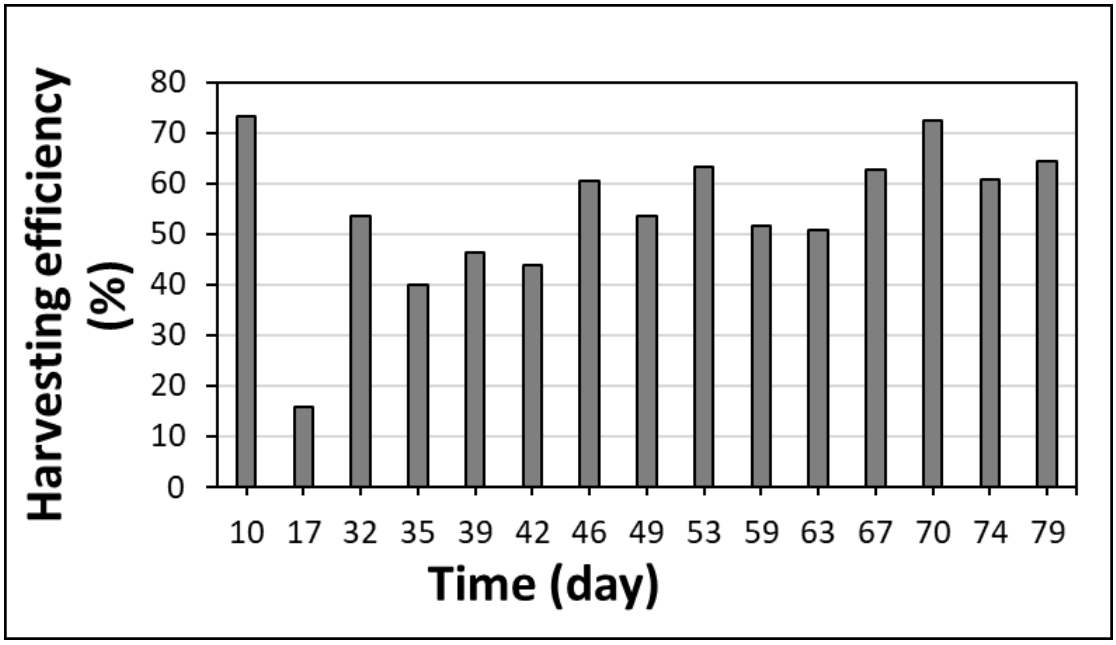

Figure 25. Harvesting efficiency based on VSS of microalgal culture. Harvesting day is counted from the first day of raceway pond operation.

6.4 Nutrient concentration

N and P removal efficiencies were determined from the pond with semi-continuous operation. The obtained results showed that *S. acuminatus* has potential to remove the nutrients.

Table 4 gives an overview of nutrient concentration change during the cultivation period. Urine dilutions represent the input concentration of nutrients for a non-filtered urine. Batch and semi-continuous ponds represent the reduction of nutrients in the pond for non-filtered samples at the end of cultivation (94 days of cultivation). The effluents show the nutrients concentrations before feeding in semi-continuous pond including 20x dilution (Effluent 10-28) and 15x dilution (Effluent 32-79). In the case of effluents, it should be considered that the concentration is not decreasing, but it is constant in certain range due to the semi-continuous removal of microalgal culture and re-feeding with diluted urine.

COD analysis showed that *S. acuminatus* consumed a certain amount of soluble COD. The original COD level decreased from 276 mg/l to 180 mg/l, representing 34 % removal of organic matter, during the whole period of 20x dilution. The COD level for 15x dilution decreased from 368 mg/ to 170 mg/l what makes 53 % removal of organic matter.

Table 4. Summary of nutrient concentrations for ponds at the end of cultivation and individual effluents (standard deviation n = 4 for diluted urine, n = 3 for ponds). All values are obtained from non-filtered samples and they are represented in mg/ l.

	P_{tot}	N_{tot}	NH_4-N	Na^+	K^+	Mg^{2+}	Ca^{2+}
20x diluetd urine	10 ± 2.6	174 ± 6.6	95 ± 37.3	34 ± 1.7	30 ± 1.9	0.11 ± 0.02	0.70 ± 0.28
15x diluted urine	12 ± 4	231 ± 9	126 ± 50	46 ± 2.3	40 ± 2.5	0.15 ± 0.001	0.92 ± 0.37
Batch Rwp (20x) [b]	8.1 ± 0.7	64.9 ±37.4	2.4 ± 1.6	92 ± 8	27 ± 2.6	4.3 ± 1.2	27 ± 1.2
Semi-cont. RwP (15x)	8.9 ± 2.0	47 ± 5.3	41.6 ± 17.0	70 ± 1.0	41 ± 2.6	4.2 ± 0.1	24.9 ± 0.3
Effluent 10 [a]	5.1	10.0	3.7	55.5	27.0	3.6	15.8
Effluent 14	6.3	26.0	3.5	57.6	26.9	3.1	17.5
Effluent 17	6.3	29.9	8.7	59.0	28.6	2.9	18.0
Effluent 21	5.5	25.0	3.2	59.3	27.5	2.8	18.5
Effluent 25	7.2	32.5	10.5	63.5	31.0	2.6	20.0
Effluent 28	13.6	43.0	23.5	66.9	34.3	3.1	21.5
Effluent 32	8.3	44.5	31.2	74.3	40.7	3.6	23.5
Effluent 35	10.6	61.9	53.9	77.4	44.9	4.0	24.6
Effluent 39	12.3	97.9	58.5	82.8	49.3	4.3	25.0
Effluent 42	10.6	47.7	37.8	60.2	41.3	4.2	26.0
Effluent 46	8.4	33.8	25.4	65.4	37.1	4.4	25.8
Effluent 49	9.4	53.1	24.4	67.1	38.2	4.3	25.7
Effluent 53	9.5	38.5	27.1	69.5	39.7	4.3	24.9
Effluent 59	9.5	43.0	31.4	69.6	39.7	4.2	24.8
Effluent 63	9.5	46.5	37.1	69.5	39.7	4.2	25.1
Effluent 67	9.0	50.0	37.6	70.4	40.9	4.4	26.2
Effluent 70	8.5	53.5	41.9	69.1	49.3	4.1	28.7
Effluent 74	9.3	61.1	45.8	70.0	41.2	4.2	23.3
Effluent 79	9.8	65.4	52.2	73.6	43.8	4.2	24.5

[a] The number of the Effluent means number of the day when it was collected, counted from the beginning of the cultivation. E.g., Effluent 10 was collected during harvesting on 10^{th} day after the beginning of the cultivation.

[b] Batch RwP (20x) refers to culture media in batch pond fed with 20x diluted urine at the end of cultivation period (similarly for semi-continuous pond).

7. DISCUSSION

7.1 Culturing of *Scenedesmus acuminatus*

S. acuminatus was chosen for this study because of its extensive use in wastewater treatment research and because of its high biomass yields (Tao et al., 2017). It was cultivated in standard N8 media for 6 months without a break to prepare enough stock culture for the whole experiment (total amount of produced *S. acuminatus* biomass in original media was 25 l). Optimal pH for most of the species is in range 7-9. pH of N8 medium for *S. acuminatus* growth was adjusted to 8.5 before inoculation but it significantly fluctuated, and it was increasing up to pH 11 during one batch cycle. An explanation for dramatically growing pH could be that CO_2 supply naturally reduces pH of the media. However, CO_2 was not supplied during the cultivation of stock *S. acuminatus* in this experiment meaning that pH rose faster. Despite high pH, *S. acuminatus* grew well with the maximum OD 6.4 in 8 days. After 8 days of culturing *S. acuminatus,* the OD started to drop from 6.4 to 5.5 - 5.9. OD 5.9 was decided as the end point of the experiment to keep microalgal cells viable and healthy without dead cells.

One reason why *S.acuminatus* could cope with so high pH is that it has a strong tolerance for pH changes. For example, Ren et al. (2013) demonstrated that new strain R-16, belonging to *Scenedesmus* species, had strong pH tolerance and could grow in pH range 4-11 with the same microalgal yield as in optimal pH (Ren et al., 2013).

7.2 Growth of S. acuminatus in different dilutions of urine

From the economical point of view, urine treatment and nutrient recovery with microalgae are the most advantageous when the urine is used as a growth medium in concentrated form. Therefore, the main objective of dilution screening was to find out the lowest possible dilution in which microalgae can effectively grow. The results showed that none of the 2x- 5x dilutions nor concentrated urine were suitable for *S. acuminatus* growth and there was a need for higher dilutions. Results from 10x- 25x dilutions showed that microalgae could grow better, but only 20x dilution showed promising results. On the other hand, microscopy revealed that cells were not in good shape in 20x dilution. The reason why 25x dilution did not support microalgal growth, is not clear because several studies tested higher dilutions than 25x and they noted microalgal growth. Jaatinen et al. (2015) examined 25x, 100x, 150x and 300x dilution and all of them supported microalgal growth. The 100x dilution promoted the highest microalgal yield (0.60 g of VSS/ l of reactor volume without trace elements) (Jaatinen et al., 2015).

So far, only study of Tuantet et al. (2014) tested microalgal growth in concentrated urine. *Chlorella sorokiniana* was cultivated in a batch system with addition of trace elements (Mg, Fe, B, Mn, Zn, Cu) to the culture media. When *C. sorokiniana* was cultivated in 5x diluted hydrolysed urine, OD_{750} increased from 0.1 to approximately 0.3 in 1 day but from second day onward OD_{750} did not show any growth. The same phenomenon was observed in concentrated urine. Tuantet et al. (2014) also tested 20x dilutions and concluded that 20x dilution showed the best growth conditions where the OD_{750} rose from 0.1 to 0.8 in 1 day and without addition of trace elements (Tuantet et al., 2014).

On the other hand, *S. acuminatus* has high potential to grow in wastewater streams even in low dilutions. Tao et al. (2017) tested the growth of *S. acuminatus* in anaerobic digestates from municipal wastewater and pulp and paper industry using dilutions from 1x – 10x. The highest biomass yield was 9.4 g of VSS/ l of reactor volume and it was growing in 1.5x diluted pulp and paper digestate (Tao et al., 2017). Interestingly, in the same study, *S. acuminatus* grew the best in 2x diluted digestate from municipal wastewater but the yield was much less, 2.2 g of VSS/ l of reactor volume (Tao et al., 2017).

Initial P concentration for pulp and paper digestate (1.5 diluted) was 16 mg of P/ l and for municipal wastewater digestate (2x diluted) 1 mg of P/ l (Tao et al., 2017). Comparing initial P concentration in this study (10 mg of P/ l in 20x diluted urine and 8 mg of P/ l in 25x diluted urine) can indicate insufficient concentration of P for achieving high microalgal yield. Moreover, P concentration decreases with hydrolyzation of urine (precipitation of P) (Tuantet et al., 2014). As it was already mentioned in Chapter 3, hydrolyzation occurs during urine storage. Therefore, one proposal for using less diluted urine for cultivation could be to avoid urine hydrolyzation. At the same time, taking into the account other ways to kill pathogens since hydrolyzation is one of the ways.

7.3 Cultivation in raceway ponds

7.3.1 Batch raceway pond cultivation

The primary objective was to determine how long the microalgal cultivation can run in the raceway pond without re-feeding with urine. Smaller raceway pond was operated as a batch system with working volume of 400 l. The preliminary results obtained from urine dilution tests indicated that 20x dilution could be the optimal to start the cultivation in raceway ponds. The cultivation began in the middle of July what ensured high daily ambient temperatures (~20 °C) and extended daylight (~19 h/ day) for enhanced grow.

The highest microalgal yield of *S. acuminatus* was 2.31 g VSS/l of pond volume (OD 6.8) at the end of August which is considered as a saturation point. After the saturation peak (40 days), the microalgal yield dropped to 2 g VSS/l of pond volume and it stayed constant until the end of the cultivation in the middle of October. It is not clear, what was the main cause of microalgal biomass reduction. Nutrient analysis showed that P was almost

depleted already after 15 days of batch operation. However, the biomass was continuously growing for following 25 days until reaching saturation peak (40 days from the beginning of batch operation). Moreover, the highest OD 6.8 obtained from raceway pond after 40 days of cultivation is comparable to growth on a standard N8 medium when the maximum OD was 6.4 in 8 days. A higher growth rate in the laboratory PBR might be caused by artificial light supply and aeration of the PBR with microalgae growing in N8 media.

Therefore, it is possible that temperature and light intensity had the biggest effect on microalgal growth and the microalgae survived based on intracellular P reserves. The measured temperature inside the pond (liquid) were almost identical with ambient temperature. The light intensity refers to daylight and actual weather condition (sunny, partly sunny or cloudy). The microalgae growing in the open pond were not supplied with any additional light illumination. Therefore, with the dropping temperature and shorter days also the microalgal production dropped because biological functions of microalgae slow down or stop in low temperatures and darkness. So, the constant OD of 5.0 could be explained by the change of the weather. Nutrient depletion could be assumed as another option for microalgal growth reduction, but for more specific conclusion there is a need for further analysis. pH fluctuation is not well understood but despite that, *S. acuminatus* cultivated in open pond proved its strong pH tolerance equally like the cultivation in standard media N8. From the visual evaluation, microalgal culture had dark green color signalizing that it is healthy, and it did not settle on the edges of the pond. Referring to the mixing in the ponds, it could be concluded that the constant velocity of 10 rpm for the volume of 400 l was sufficient due to proper algal growth.

There is no evidence in the scientific literature about the microalgae grown on source separated urine in pilot scale. Nevertheless, a very similar study was conducted by Posadas et al. (2015) where they presented a pilot scale of *Scenedesmus* cultivation in open raceway ponds. The main goal of their study was to treat secondary domestic wastewater by growing microalgae and to test the impact of the CO_2 source (pure CO_2 or CO_2 from flue gas) on microalgal performance (biomass yield and composition). The working volumes of RwP were 700 l, 800 l, and 850 l and they were operated from August until December as a batch system. The microalgal yield obtained from the study Posadas et al. (2015) is comparable to microalgal yield in this study: highest yield was 0.5 g of TSS/ l of pond volume with the addition of pure CO_2 during 1-month cultivation (August-September). During the period from September- November RwP were supplied with flue gas, and the microalgal yield was 0.4 g of TSS/ l of pond volume (Posadas et al., 2015).

This study brings new knowledge about the potential feasibility and scalability of the urine treated by microalgae. Unlike Posadas et al. (2015) study, this study showed that microalgae could grow not only without additional study supply but also in colder climate since Posadas et al. (2015) pilot scale was performed in Spain. However, this study is lacking recycling of flue gases what could be attractive option coupling microalgal cultivation with decreased greenhouse gas emission.

7.3.2 Semi-continuous raceway pond cultivation

Bigger raceway pond was operated as a semi-continuous system with working volume of 2000 l. The main objective was to test and demonstrate that *S. acuminatus* can continuously grow in urine and remove nutrients from the urine. For the first cycle, 20x dilution was tested. In summary, *S. acuminatus* was consistently increasing, and the visual evaluation, as well as microscopy, showed that the cells were in good condition (forming colonies) with dark green color despite significant pH changes (8.4 - 11). Therefore, the dilution was changed to 15x dilution after one month to test the influence of less diluted urine on microalgal growth. Throughout the pond operation, very little contamination was noticed with microscopy which was caused by growing zooplankton.

When the dilution was changed, microalgae could still grow and achieve the highest yield during the whole cultivation, 0.45 g VSS/l of pond volume. However, after second feeding with 15x dilution, microalgal biomass was rapidly reduced what could be noticed from OD measurement, microscopic examination and also from the brown color of the culture in the pond. Rapid microalgal reduction could be due to the suddenly elevated concentration of ammonia (NH_3) in the 15x diluted urine compared to 20x diluted urine. Consequently, NH_3 could induce toxic shock on microalgae by passing through the microalgal membrane because of its uncharged and lipid soluble character (Collos and Harrison, 2014). Nevertheless, Collos and Harrison (2014) reported that microalgal class Chlorophyceae, where *S. acuminatus* belongs to, can have a strong tolerance for high ammonia concentration (up to 630 mg/l) (Collos and Harrison, 2014). This opinion does not support the idea that 15x diluted urine in this work contains a toxic level of NH_3 (<60 mg/l what is an estimation based on NH_4-N concentration summarized in Table 4).

To recover microalgal biomass in the pond, the feeding after harvesting consisted only from the addition of tap water for following two weeks. Water could neutralize ammonia in the culture, and after two weeks, when microalgae were stabilized (microscopic evaluation of microalgae and visual color evaluation of the culture in the pond), the feeding with urine (15x) was again applied. pH was in the optimal range for microalgal growth, but the color of microalgal culture was still little bit brownish. In addition, microalgal biomass was settling very fast on the edge of the pond what could be caused by the insufficient mixing of 2000 l. On the other hand, when the mixing velocity was increased higher than 13 rpm, the culture with microalgae was splashing. This problem could be potentially solved in the future by adding one more paddle wheel.

Continuous raceway pond was running for 3 months from the middle of July to the middle of October. The temperature and light intensity influenced the microalgal growth, meaning higher ambient temperature (~20 °C) and long daylight (~19 h/day) enhanced the growth, but with dropped values of temperature and light intensity, also microalgal growth was less significant. Consequently, reduced microalgal growth can explain stable pH (~8.5) at lower dilution. The nutrient analysis showed that the microalgal culture still

contained nutrients for microalgal growth at the end of cultivation and the NH_3 levels were not toxic. Therefore, temperature, short daylight and constant removal of microalgal biomass seem to have the biggest impact on a microalgal inability to achieve OD_{660} higher than 0.8.

One of the challenges in this project was the harvesting of microalgal biomass twice a week. The harvesting consisted of simple filtration. From the results of harvesting efficiency, it can be seen that most of the biomass (~50 %) was harvested during each harvesting, but a big part was drained with the effluent. Nevertheless, screening for efficient harvesting method was not the main objective of this research.

A similar study was conducted by Adamsson (2000). *S. acuminatus* was grown on 50x diluted urine in 130 l cylindrical tanks under semi-batch operation in a greenhouse. The cultivation started in May, and it lasted until September. Artificial light was not supplied, meaning only light supply was provided by the diurnal cycle, and the temperature was dependent on the outside climate. Fresh human urine was collected and stored. Cold tap water was used for dilution of the urine, and it was supplemented with trace elements. The tanks were aerated, and the culture was stirred with magnetic stirrer. The algal biomass was harvested 3 times per week (10 l), and it was replaced with fresh 50x diluted urine (HRT 8 days). The highest microalgal yield resulted in 287 mg of dry weight/ l of tank volume in 12 weeks (Adamsson, 2000).

Comparing to the study of Adamsson (2000), this study gave further indications that *S. acuminatus* can achieve same biomass yield in bigger working volume in almost two times less diluted urine what could bring economic benefits in treating urine.

7.4 Nutrient recovery

Scenedesmus sp. is widely used in different studies for its ability to recover nutrients from different wastewater streams, for example, pulp and paper industry or municipal wastewater (Tao et al.,2017). In this study, *S. acuminatus* showed potential to remove nutrients from diluted urine. It has to be noted that obtained concentrations of nutrients are from non-filtered samples. Thus, not all removed nutrients are necessarily recovered by microalgae (present inside the cell), but they can be present also as a precipitate.

P is present mostly as the phosphate ion in urine what is the only form that the microalgae can uptake. Other forms present in the urine, are precipitated P salts (e.g., struvite) formed when the pH is high. Consequently, part of the precipitated salts could settle on the bottom of the urine tank (already during the urine storage) causing reduction of phosphate ion concentration during the pumping of the urine out of the tank. Struvite precipitation is responsible also for ammonium reduction since it precipitates along with P. Almost half of the N is present in the form of ammonium ions which is the most preferred for microalgal uptake. The decrease of ammonium concentration can also be caused by ammonia

volatilization since high pH (> 8.5) of the culture leads to the conversion of ammonia to gaseous ammonium.

Regarding the trace elements in the urine, cation analysis showed that the urine contains very low amount of Ca^{2+} and does not contain any Mg^{2+}. Precipitation of Mg^{2+} and Ca^{2+} ions at the bottom of urine collection tank could be one possibility why they are not present in urine. Another possible reason could be that the urine was collected from dry toilets. Flushing the urine with water from flush toilets can increase Mg^{2+} and Ca^{2+} levels in the urine (Maurer et al., 2006). Surprisingly, analysis of the ponds and effluents showed that the concentration of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} are higher than in urine, but the reason was not studied in detail.

However, the concentrated urine sample was collected from the urine collection tank at the beginning of the experiment. This particular sample was used for the nutrient analysis in the urine as well as the calculation of nutrient content in 15x and 20x diluted urine (input nutrient concentration). Precipitates could settle at the bottom of the bottle, but the bottle was mixed before nutrient analysis. On the other hand, the pump did not touch the bottom of the tank when pumping the urine into the pond. Thus, precipitates could stay at the bottom of the tank.

Importantly, the primary purpose of this study was to investigate nutrient recovery by microalgae *S. acuminatus*. The precipitation was not considered as a main mechanism of nutrient recovery and its role in this study is not clear, but it could affect obtained results of the input: output nutrient ratio and actual nutrient uptake by microalgae. Nevertheless, microalgal nutrient recovery could be supposed from the stable results of P and N nutrient concentration in the effluents. In other words, harvested biomass was replaced by new culture media (diluted urine) causing accumulation of new nutrients. Consequently, the newly grown microalgal biomass could consume the excess of nutrients, keeping constant concentration of P and N in the effluent.

So far, only study of Adamsson (2000) reported N and P recovery by microalgae in bigger scale than the laboratory scale. Filtered samples of *S. acuminatus* grown in 50x diluted urine showed that N removal efficiency was 67 % and P removal efficiency was 36 % in 4 months (May-August), but it was 98 % and 97 % respectively in 2 months (September-October). It is important to take into the account that the study of Adamsson (2000) experimented growing algae in urine which were used as a feed for zooplankton and zooplankton was consequently used as a fertilizer for tomatoes. Therefore, the calculation of total nutrient recovery is based on this cultivation chain, meaning not all nutrients were consumed only by *S. acuminatus* but also by zooplankton and tomatoes (Adamsson, 2000).

The COD removal (34 %) for 20x dilution indicates that the microalgal culture was predominantly photoautotrophic and it was utilizing mainly CO_2 as a carbon source. In 15x

dilution, microalgal culture could be both mixotrophic and photoautotrophic (53 % removal). High COD removal can also be caused by the bacterial community that is consuming organic matter. However, the microscopic evaluation did not reveal severe bacterial contamination. A similar experience of mixed *S. acuminatus* culture, mixotrophic and heterotrophic, observed Tao et al. (2017) when soluble COD was removed up to 39 % from the anaerobic digestates (Tao et al., 2017).

From the presented results can be concluded that the removal of P and N from urine was accomplished, but detailed study and analysis of filtered samples and biomass composition need to be carried to determine P and N recovery by microalgae.

8. CONCLUSION

This study showed that 20x and 15x diluted urine could provide enough nutrients and support microalgal growth in open ponds. This pilot project also revealed that it is feasible to grow microalgae in colder climate such as Nordic countries have.

S. acuminatus was able to grow on 20x diluted urine as a batch system for 94 days without any addition of extra urine, trace elements, artificial light or CO_2 supply. The result of microalgal cultivation in the pond was comparable to the result of microalgal cultivation in the standard media, OD_{660} 6.8 and 6.4 respectively, and the highest biomass yield was 2.31 g VSS/ l of pond volume. Higher ambient temperatures (20°C), as well as long daylight (19 h/ day), enhanced microalgal growth, and with the reduction of these two external factors in combination with nutrient depletion, also the microalgal growth was reduced. Furthermore, this study showed that it is possible to keep a semi-continuous growth of *S. acuminatus* cultivated on diluted urine (15x and 20x) in open ponds for more than 94 days (maximum OD_{660} for 15x dilution was 0.8). *S. acuminatus* showed an ability to remove phosphorus and nitrogen as well as organic matter from the diluted urine. Nevertheless, *S. acuminatus* grown in open ponds was influenced by many external factors such as temperature, light intensity or nutrient composition of the cultivation medium. To describe more precisely how the combination of these factors affected the behavior of *S. acuminatus*, more analysis, and more data are needed.

Ideas for improvement of the microalgal cultivation in open ponds and nutrient recovery could include testing higher urine dilutions, using more efficient harvesting methods and potential re-use of the effluent from the harvesting for re-feeding the pond instead of using tap water. Lastly, plans could focus on further microalgal biomass use to close the loop of the circular economy.

REFERENCES

ABC Science. (2012). *Urine diversion toilet.* Retrieved 10/31, 2017, from http://www.abc.net.au/science/articles/2012/01/31/3415550.htm

Al Hattab, M., Ghaly, A., & Hammoud, A. (2015). Microalgae Harvesting Methods for Industrial Production of Biodiesel: Critical Review and Comparative Analysis. *Journal of Fundamentals of Renewable Energy and Applications*, *5*(2), 1000154. https://doi.org/10.4172/20904541.1000154

Antonini, S., Nguyen, P. T., Arnold, U., Eichert, T., & Clemens, J. (2012). Solar thermal evaporation of human urine for nitrogen and phosphorus recovery in Vietnam. *Science of the Total Environment*, *414*, 592–599. https://doi.org/10.1016/j.scitotenv.2011.11.055

APHA. (1998). Stadard methods for the examination of water and wastewater. Available: https://www.standardmethods.org/. Accessed 11/2017.

Azad, H. S., & Borchardt, J. A. (1970). Variations in phosphorus uptake by algae. *Environmental Science & Technology*, *4*(9), 737–743. https://doi.org/10.1021/es60044a008

Beler Baykal, B., Kocaturk, N. P., Allar, A. D., & Sari, B. (2009). The effect of initial loading on the removal of ammonium and potassium from source-separated human urine via clinoptilolite. *Water Science and Technology*, *60*(10), 2515 LP-2520. Retrieved from http://wst.iwaponline.com/content/60/10/2515.abstract

Brennan, L., & Owende, P. (2010, February 1). Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*. Pergamon. https://doi.org/10.1016/j.rser.2009.10.009

Cai, T., Park, S. Y., & Li, Y. (2013, March 1). Nutrient recovery from wastewater streams by microalgae: Status and prospects. *Renewable and Sustainable Energy Reviews*. Pergamon. https://doi.org/10.1016/j.rser.2012.11.030

Canfield, D. E., Glazer, A. N., & Falkowski, P. G. (2010). The evolution and future of Earth's nitrogen cycle. *Science (New York, N.Y.)*, *330*(6001), 192–6. https://doi.org/10.1126/science.1186120

Carey, D. E., Yang, Y., McNamara, P. J., & Mayer, B. K. (2016, September 1). Recovery of agricultural nutrients from biorefineries. *Bioresource Technology*. Elsevier. https://doi.org/10.1016/j.biortech.2016.02.093

Chang, Y., Wu, Z., Bian, L., Feng, D., & Leung, D. Y. C. (2013). Cultivation of Spirulina platensis for biomass production and nutrient removal from synthetic human urine. *Applied Energy*, *102*, 427–431. https://doi.org/10.1016/j.apenergy.2012.07.024

Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J., & Chang, J. S. (2011). Cultivation,

photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology*, *102*(1), 71–81. https://doi.org/10.1016/j.biortech.2010.06.159

Childers, D. L., Corman, J., Edwards, M., & Elser, J. J. (2011). Sustainability Challenges of Phosphorus and Food: Solutions from Closing the Human Phosphorus Cycle. *BioScience*, *61*(2), 117–124. https://doi.org/10.1525/bio.2011.61.2.6

Chislock, M. F., Doster, E., Zitomer, R. A., & Wilson, A. E. (2013). Eutrophication: Causes, Consequences, and Controls in Aquatic Ecosystems | Learn Science at Scitable. Retrieved November 18, 2017, from https://www.nature.com/scitable/knowledge/library/eutrophication-causes-consequences-and-controls-in-aquatic-102364466

Cieślik, B., & Konieczka, P. (2017, January 20). A review of phosphorus recovery methods at various steps of wastewater treatment and sewage sludge management. The concept of "no solid waste generation" and analytical methods. *Journal of Cleaner Production*. Elsevier. https://doi.org/10.1016/j.jclepro.2016.11.116

Collos, Y., & Harrison, P. J. (2014, March 15). Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Marine Pollution Bulletin*. Pergamon. https://doi.org/10.1016/j.marpolbul.2014.01.006

Coppens, J., Lindeboom, R., Muys, M., Coessens, W., Alloul, A., Meerbergen, K., … Vlaeminck, S. E. (2016). Nitrification and microalgae cultivation for two-stage biological nutrient valorization from source separated urine. *Bioresource Technology*, *211*, 41–50. https://doi.org/10.1016/j.biortech.2016.03.001

Cuellar-Bermudez, S. P., Aleman-Nava, G. S., Chandra, R., Garcia-Perez, J. S., Contreras-Angulo, J. R., Markou, G., … Parra-Saldivar, R. (2017). Nutrients utilization and contaminants removal. A review of two approaches of algae and cyanobacteria in wastewater. *Algal Research*, *24*, 438–449. https://doi.org/10.1016/j.algal.2016.08.018

Delgadillo-Mirquez, L., Lopes, F., Taidi, B., & Pareau, D. (2016). Nitrogen and phosphate removal from wastewater with a mixed microalgae and bacteria culture. *Biotechnology Reports*, *11*, 18–26. https://doi.org/10.1016/j.btre.2016.04.003

Dineshkumar, R., Kumaravel, R., Gopalsamy, J., Sikder, M. N. A., & Sampathkumar, P. (2017). Microalgae as Bio-fertilizers for Rice Growth and Seed Yield Productivity. *Waste and Biomass Valorization*, 1–8. https://doi.org/10.1007/s12649-017-9873-5

Ganrot, Z., Dave, G., & Nilsson, E. (2007). Recovery of N and P from human urine by freezing, struvite precipitation and adsorption to zeolite and active carbon. *Bioresource Technology*, *98*(16), 3112–3121. https://doi.org/https://doi.org/10.1016/j.biortech.2006.10.038

Grundestam, J., & Hellström, D. (2007). Wastewater treatment with anaerobic membrane bioreactor and reverse osmosis. *Water Science & Technology*, *56*(5), 211. https://doi.org/10.2166/wst.2007.574

HACH. (2017). LCK Cuvette Test System. Available: https://uk.hach.com/lck. Accessed 11/2017.

Jaatinen, S., Lakaniemi, A.-M., & Rintala, J. (2015). Use of diluted urine for cultivation of Chlorella vulgaris. *Environmental Technology*, *3330*(November), 1–36. https://doi.org/10.1080/09593330.2015.1105300

Kvarnström, E., & Stockholm Environment Institute. (2006). *Urine diversion : one step towards sustainable sanitation*. Stockholm Environment Institute. Retrieved from https://books.google.fi/books?hl=sk&lr=&id=3JfWwl8iE10C&oi=fnd&pg=PR6&dq=Urine+diversion:+One+step+towards+sustainable+sanitation&ots=JroDf1_q2s&sig=1dhedX5meFs1HMnq7--VYfnWwxE&redir_esc=y#v=onepage&q=Urine diversion%3A One step towards sustainable sanitation&f=false

Langergraber, G., & Muellegger, E. (2005, April 1). Ecological Sanitation - A way to solve global sanitation problems? *Environment International*. Pergamon. https://doi.org/10.1016/j.envint.2004.08.006

Ledezma, P., Kuntke, P., Buisman, C. J. N., Keller, J., & Freguia, S. (2015, April 1). Source-separated urine opens golden opportunities for microbial electrochemical technologies. *Trends in Biotechnology*. Elsevier Current Trends. https://doi.org/10.1016/j.tibtech.2015.01.007

Lehtovuori, P., Edelman, H., Rintala, J., Jokinen, A., Rantanen, A., Särkilahti, M., & Joensuu, T. (n.d.). DEVELOPMENT VISION FOR HIEDANRANTA. Retrieved from https://tutcris.tut.fi/portal/files/5897526/Development_vision_for_Hiedanranta.pdf

Li, C., Cabassud, C., Reboul, B., & Guigui, C. (2015). Effects of pharmaceutical micropollutants on the membrane fouling of a submerged MBR treating municipal wastewater: Case of continuous pollution by carbamazepine. *Water Research*, *69*, 183–194. https://doi.org/10.1016/j.watres.2014.11.027

Lomas, M. W., & Glibert, P. M. (1999). Temperature regulation of nitrate uptake: A novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnology and Oceanography*, *44*(3), 556–572. https://doi.org/10.4319/lo.1999.44.3.0556

Maurer, M., Pronk, W., & Larsen, T. A. (2006, October 1). Treatment processes for source-separated urine. *Water Research*. Pergamon. https://doi.org/10.1016/j.watres.2006.07.012

Melia, P. M., Cundy, A. B., Sohi, S. P., Hooda, P. S., & Busquets, R. (2017). Trends in the recovery of phosphorus in bioavailable forms from wastewater. *Chemosphere*, *186*(Supplement C), 381–395. https://doi.org/https://doi.org/10.1016/j.chemosphere.2017.07.089

Milton, R. D., Cai, R., Abdellaoui, S., Leech, D., De Lacey, A. L., Pita, M., & Minteer, S. D. (2017). Bioelectrochemical Haber-Bosch Process: An Ammonia-Producing H2 /N2 Fuel Cell. *Angewandte Chemie International Edition*, *56*(10), 2680–2683. https://doi.org/10.1002/anie.201612500

Patel, A., Gami, B., Patel, P., & Patel, B. (2017, May 1). Microalgae: Antiquity to era of

integrated technology. *Renewable and Sustainable Energy Reviews*. Pergamon. https://doi.org/10.1016/j.rser.2016.12.081

Posadas, E., Del, M., Morales, M., Gomez, C., Acién, F. G., & Muñoz, R. (2015). Influence of pH and CO 2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways. *CHEMICAL ENGINEERING JOURNAL*, *265*, 239–248. https://doi.org/10.1016/j.cej.2014.12.059

Powell, N., Shilton, A. N., Pratt, S., & Chisti, Y. (2008). Factors Influencing Luxury Uptake of Phosphorus by Microalgae in Waste Stabilization Ponds. *Environmental Science & Technology*, *42*(16), 5958–5962. https://doi.org/10.1021/es703118s

Rawat, I., Ranjith Kumar, R., Mutanda, T., & Bux, F. (2011). Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Applied Energy*, *88*(10), 3411–3424. https://doi.org/10.1016/j.apenergy.2010.11.025

Reay, D. S., Nedwell, D. B., Priddle, J., & Ellis-Evans, J. C. (1999). Temperature dependence of inorganic nitrogen uptake: reduced affinity for nitrate at suboptimal temperatures in both algae and bacteria. *Applied and Environmental Microbiology*, *65*(6), 2577–84. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10347046

Ren, H.-Y., Liu, B.-F., Ma, C., Zhao, L., & Ren, N.-Q. (2013). A new lipid-rich microalga Scenedesmus sp. strain R-16 isolated using Nile red staining: effects of carbon and nitrogen sources and initial pH on the biomass and lipid production. *Biotechnology for Biofuels*, *6*(1), 143. https://doi.org/10.1186/1754-6834-6-143

Roy, E. D. (2017). Phosphorus recovery and recycling with ecological engineering: A review. *Ecological Engineering*, *98*, 213–227. https://doi.org/10.1016/j.ecoleng.2016.10.076

Schmidt, J. J., Gagnon, G. A., & Jamieson, R. C. (2016). Microalgae growth and phosphorus uptake in wastewater under simulated cold region conditions. *Ecological Engineering*, *95*, 588–593. https://doi.org/10.1016/j.ecoleng.2016.06.114

Schönning, C., Stenström, T. A., & Programme, E. (n.d.). Guidelines on the Safe Use of Urine and Faeces in Ecological Sanitation Systems. Retrieved from www.sei.se

Simha, P., & Ganesapillai, M. (2017, May 1). Ecological Sanitation and nutrient recovery from human urine: How far have we come? A review. *Sustainable Environment Research*. Elsevier. https://doi.org/10.1016/j.serj.2016.12.001

Solovchenko, A., Verschoor, A. M., Jablonowski, N. D., & Nedbal, L. (2016, September 1). Phosphorus from wastewater to crops: An alternative path involving microalgae. *Biotechnology Advances*. Elsevier. https://doi.org/10.1016/j.biotechadv.2016.01.002

Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering*, *101*(2), 87–96. https://doi.org/10.1263/jbb.101.87

Tampere. (2017). Available: https://www.tampere.fi/asuminen-ja-ymparisto/kaupunkisuunnittelu-ja-rakentamishankkeet/hiedanranta.html. Accessed: 10/2017

Tao, R., Kinnunen, V., Praveenkumar, R., Lakaniemi, A. M., & Rintala, J. A. (2017). Comparison of Scenedesmus acuminatus and Chlorella vulgaris cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge. *Journal of Applied Phycology*, (September), 1–12. https://doi.org/10.1007/s10811-017-1175-6

Tao, R., Lakaniemi, A. M., & Rintala, J. A. (2017). Cultivation of Scenedesmus acuminatus in different liquid digestates from anaerobic digestion of pulp and paper industry biosludge. *Bioresource Technology*, *245*, 706–713. https://doi.org/10.1016/j.biortech.2017.08.218

Tarpeh, W. A., Udert, K. M., & Nelson, K. L. (2017). Comparing Ion Exchange Adsorbents for Nitrogen Recovery from Source-Separated Urine. *Environmental Science & Technology*, *51*(4), 2373–2381. https://doi.org/10.1021/acs.est.6b05816

Tindall, J. A., Weeks, E. P., & Friedel, M. (2005). *Part 2: A field study of enhanced remediation of Toluene in the vadose zone using a nutrient solution*. *Water, Air, and Soil Pollution* (Vol. 168). https://doi.org/10.1007/s11270-005-3584-4

Trivedi, J., Aila, M., Bangwal, D. P., Kaul, S., & Garg, M. O. (2015). Algae based biorefinery - How to make sense? *Renewable and Sustainable Energy Reviews*, *47*, 295–307. https://doi.org/10.1016/j.rser.2015.03.052

Tuantet, K., Janssen, M., Temmink, H., Zeeman, G., Wijffels, R. H., & Buisman, C. J. N. (2014). Microalgae growth on concentrated human urine. *Journal of Applied Phycology*, *26*(1), 287–297. https://doi.org/10.1007/s10811-013-0108-2

Tuantet, K., Temmink, H., Zeeman, G., Janssen, M., Wijffels, R. H., & Buisman, C. J. N. (2014). Nutrient removal and microalgal biomass production on urine in a short light-path photobioreactor. *Water Research*, *55*, 162–174. https://doi.org/10.1016/j.watres.2014.02.027

Wang, L., Li, Y., Sommerfeld, M., & Hu, Q. (2013). A flexible culture process for production of the green microalga Scenedesmus dimorphus rich in protein, carbohydrate or lipid. *Bioresource Technology*, *129*, 289–295. https://doi.org/10.1016/j.biortech.2012.10.062

Wang, L., Yang, H., Wang, Q., & Niranjan, S. (2013). Energy and Environment: Challenges and Achievements in Rapid Urbanization. *The Scientific World Journal*, *2013*, 1–2. https://doi.org/10.1155/2013/594816

Wang, Z., Gong, H., Zhang, Y., Liang, P., & Wang, K. (2017). Nitrogen recovery from low-strength wastewater by combined membrane capacitive deionization (MCDI) and ion exchange (IE) process. *Chemical Engineering Journal*, *316*, 1–6. https://doi.org/10.1016/j.cej.2017.01.082

Wayama, M., Ota, S., Matsuura, H., Nango, N., Hirata, A., & Kawano, S. (2013). Three-dimensional ultrastructural study of oil and astaxanthin accumulation during

encystment in the green alga Haematococcus pluvialis. *PloS One*, *8*(1), e53618. https://doi.org/10.1371/journal.pone.0053618

Whitton, R., Le Mével, A., Pidou, M., Ometto, F., Villa, R., & Jefferson, B. (2016). Influence of microalgal N and P composition on wastewater nutrient remediation. *Water Research*, *91*, 371–378. https://doi.org/10.1016/j.watres.2015.12.054

Yang, Y.-Y., Toor, G. S., Wilson, P. C., & Williams, C. F. (2017). Micropollutants in groundwater from septic systems: Transformations, transport mechanisms, and human health risk assessment. https://doi.org/10.1016/j.watres.2017.06.054

Zhang, S., Lim, C. Y., Chen, C. L., Liu, H., & Wang, J. Y. (2014). Urban nutrient recovery from fresh human urine through cultivation of Chlorella sorokiniana. *Journal of Environmental Management*, *145*, 129–136. https://doi.org/10.1016/j.jenvman.2014.06.013

Zhu, L., Dong, D., Hua, X., Xu, Y., Guo, Z., & Liang, D. (2017). Ammonia nitrogen removal and recovery from acetylene purification wastewater by air stripping. *Water Science and Technology*, *75*(11), 2538–2545. https://doi.org/10.2166/wst.2017.117